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# CHEMICAL EMBRYOLOGY

IN THREE VOLUMES

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ZEUS LIBERATING LIVING BEINGS FROM AN EGG

The Frontispiece of William Harvey's book on the *Generation of Animals*, 1651

# CHEMICAL EMBRYOLOGY

BY

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# CONTENTS

## OF THE THREE VOLUMES

VOLUME I

Prolegomena *page* 2

PART I

The Theory of Chemical Embryology

Philosophy, Embryology, and Chemistry 7
The Historical Perspective 10
Obstacles to Chemical Embryology 13
The Stumbling-block of Hormism 14
Finalism as a Rock of Offence 16
Organicism as an Occasion of Falling 25
Organicism and Emergence 30
Neo-Mechanism as a Theory for Chemical Embryology 32

PART II

The Origins of Chemical Embryology

Preliminary Note 41
Section 1. Embryology in Antiquity 44
1·1. Non-Hellenic Antiquity 44
1·2. Hellenic Antiquity; the Pre-Socratics 50
1·3. Hippocrates; the Beginning of Observation 53
1·4. Aristotle 59
1·5. The Hellenistic Age 77
1·6. Galen 85
Section 2. Embryology from Galen to the Renaissance 91
2·1. Patristic, Talmudic, and Arabian Writers 91
2·2. St Hildegard; the Lowest Depth 95
2·3. Albertus Magnus 97
2·4. The Scholastic Period 103
2·5. Leonardo da Vinci 107
2·6. The Sixteenth Century; the Macro-iconographers 110

Section 3. Embryology in the Seventeenth and Eighteenth Centuries *page* 125
3·1. The Opening Years of the Seventeenth Century 125
3·2. Kenelm Digby and Nathaniel Highmore 129
3·3. Thomas Browne and the Beginning of Chemical Embryology 135
3·4. William Harvey 138
3·5. Gassendi and Descartes; Atomistic Embryology 156
3·6. Walter Needham and Robert Boyle 160
3·7. Marcello Malpighi; Micro-iconography and Preformationism 166
3·8. Robert Boyle and John Mayow 169
3·9. The Theories of Foetal Nutrition 176
3·10. Boerhaave, Hamberger, Mazin 182
3·11. Albrecht v. Haller and his Contemporaries 188
3·12. Ovism and Animalculism 199
3·13. Preformation and Epigenesis 205
3·14. The Close of the Eighteenth Century 215
3·15. The Beginning of the Nineteenth Century 220

PART III

## General Chemical Embryology

Preliminary Note 231
Section 1. The Unfertilised Egg as a Physico-chemical System 232
1·1. Introduction 232
1·2. General Characteristics of the Avian Egg 232
1·3. The Proportion of Parts in the Avian Egg 236
1·4. Chemical Constitution of the Avian Egg as a Whole 242
1·5. The Shell of the Avian Egg 255
1·6. The Avian Egg-white 265
1·7. The Avian Yolk 280
1·8. The Avian Yolk-proteins 287
1·9. The Fat and Carbohydrate of the Avian Yolk 294
1·10. The Ash of the Avian Egg 302
1·11. General Characteristics of non-Avian Eggs 306
1·12. Egg-shells and Egg-membranes 321
1·13. Proteins and other Nitrogenous Compounds 331
1·14. Fats, Lipoids, and Sterols 346
1·15. Carbohydrates 355
1·16. Ash 357

Section 2. On Increase in Size and Weight *page* 368
2·1. Introduction 368
2·2. The Existing Data 369
2·3. The General Nature of Embryonic Growth 383
2·4. The Empirical Formulae 389
2·5. Percentage Growth-rate and the Mitotic Index 399
2·6. Yolk-absorption Rate 405
2·7. The Autocatakinetic Formulae 408
2·8. Instantaneous Percentage Growth-rate 420
2·9. Growth Constants 434
2·10. The Growth of Parts 440
2·11. Variability and Correlation 455
2·12. Explantation and the Growth-promoting Factor 460
2·13. Incubation Time and Gestation Time 470
2·14. The Effect of Heat on Embryonic Growth 498
2·15. Temperature Coefficients 503
2·16. Temperature Characteristics 515
2·17. The Effect of Light on Embryonic Growth 533
2·18. The Effect of X-rays and Electricity on Embryonic Growth 536
2·19. The Effect of Hormones on Embryonic Growth 538

Section 3. On Increase in Complexity and Organisation 541
3·1. The Independence of Growth and Differentiation 541
3·2. Differentiation-rate 544
3·3. Chemical Processes and Organic Form 552
3·4. The Types of Morphogenetic Action 559
3·5. Pluripotence and Totipotence 567
3·6. Self-differentiation and Organiser Phenomena 570
3·7. Functional Differentiation 580
3·8. Axial Gradients 582
3·9. Organised and Unorganised Growth 606
3·10. Chemical Embryology and Genetics 608

VOLUME II

Section 4. The Respiration and Heat-production of the Embryo 615
4·1. Early Work on Embryonic Respiration 615
4·2. Respiration of Echinoderm Embryos in General 623
4·3. Rhythms in Respiratory Exchange 641

Section 4·4. Heat Production and Calorific Quotients of Echinoderm Embryos *page* 649
4·5. Respiration of Annelid, Nematode, Rotifer, and Mollusc Embryos 659
4·6. Respiration of Fish Embryos 665
4·7. Respiration of Amphibian Embryos 671
4·8. Heat-production of Amphibian Embryos 682
4·9. Respiration of Insect Embryos 687
4·10. Respiration of Reptile Embryos 692
4·11. Respiration of Avian Embryos in General 693
4·12. Heat-production of Avian Embryos 704
4·13. Later Work on the Chick's Respiratory Exchange 708
4·14. The Air-space and the Shell 719
4·15. Respiration of Mammalian Embryos 726
4·16. Heat-production of Mammalian Embryos 732
4·17. Anaerobiosis in Embryonic Life 742
4·18. Metabolic Rate in Embryonic Life 746
4·19. Respiratory Intensity of Embryonic Cells *in vitro* 755
4·20. Embryonic Tissue-respiration and Glycolysis 758
4·21. The Genesis of Heat Regulation 772
4·22. Light-production in Embryonic Life 776

Section 5. Biophysical Phenomena in Ontogenesis 777
5·1. The Osmotic Pressure of Amphibian Eggs 777
5·2. The Genesis of Volume Regulation 786
5·3. The Osmotic Pressure of Aquatic Arthropod Eggs 790
5·4. The Osmotic Pressure of Fish Eggs 793
5·5. Osmotic Pressure and Electrical Conductivity in Worm and Echinoderm Eggs 799
5·6. The Osmotic Pressure of Terrestrial Eggs 812
5·7. Specific Gravity 820
5·8. Potential Differences, Electrical Resistance, Blaze Currents and Cataphoresis 825
5·9. Refractive Index, Surface Tension and Viscosity 833

Section 6. General Metabolism of the Embryo 839
6·1. The *p*H of Aquatic Eggs 839
6·2. The *p*H of Terrestrial Eggs 855
6·3. *r*H in Embryonic Life 865
6·4. Water-metabolism of the Avian Egg 870

Section 6·5. Water-content and Growth-rate *page* 883
6·6. Water-absorption and the Evolution of the Terrestrial Egg 889
6·7. Water-metabolism in Aquatic Eggs 906
6·8. The Chemical Constitution of the Embryonic Body in Birds and Mammals 911
6·9. Absorption-mechanisms and Absorption-intensity 917
6·10. Storage and Combustion; the Plastic Efficiency Coefficient 934
6·11. Metabolism of the Avian Spare Yolk 939
6·12. Maternal Diet and Embryonic Constitution 943

Section 7. The Energetics and Energy-sources of Embryonic Development 946
7·1. The Energy Lost from the Egg during Development 946
7·2. Energy of Growth and Energy of Differentiation 956
7·3. The Relation between Energy Lost and Energy Stored 962
7·4. Real Energetic Efficiency 969
7·5. Apparent Energetic Efficiency 972
7·6. Synthetic Energetic Efficiency 981
7·7. The Sources of the Energy Lost from the Egg 986

Section 8. Carbohydrate Metabolism 1000
8·1. General Observations on the Avian Egg 1000
8·2. Total Carbohydrate, Free Glucose, and Glycogen 1001
8·3. Ovomucoid and Combined Glucose 1007
8·4. Carbohydrate and Fat 1014
8·5. The Metabolism of Glycogen and the Transitory Liver 1018
8·6. Free Glucose, Glycogen, and Insulin in the Embryonic Body 1029
8·7. General Scheme of Carbohydrate Metabolism in the Avian Egg 1035
8·8. Embryonic Tissue Glycogen 1036
8·9. Embryonic Blood Sugar 1039
8·10. Carbohydrate Metabolism in Amphibian Development 1043
8·11. Carbohydrate Metabolism of Invertebrate Eggs 1047
8·12. Pentoses 1051
8·13. Lactic Acid 1051
8·14. Fructose 1054

Section 9. Protein Metabolism 1055
9·1. The Structure of the Avian Egg-proteins before and after Development 1055
9·2. Metabolism of the Individual Amino-Acids 1059
9·3. The Relations between Protein and non-Protein Nitrogen 1065
9·4. The Accumulation of Nitrogenous Waste Products 1076

Section 9·5. Protein Catabolism *page* 1086
9·6. Nitrogen-excretion; Mesonephros, Allantois, and Amnios 1095
9·7. The Origin of Protective Syntheses 1101
9·8. Protein Metabolism of Reptilian Eggs 1103
9·9. Protein Metabolism of Amphibian Eggs 1104
9·10. Protein Metabolism in Teleostean Ontogeny 1111
9·11. Protein Metabolism in Selachian Ontogeny 1114
9·12. Protein Metabolism of Insect, Worm, and Echinoderm Eggs 1118
9·13. Protein Utilisation in Mammalian Embryonic Life 1122
9·14. Protein Utilisation of Explanted Embryonic Cells 1130
9·15. Uricotelic Metabolism and the Evolution of the Terrestrial Egg 1132

Section 10. The Metabolism of Nucleins and Nitrogenous Extractives 1146
10·1. Nuclein Metabolism of the Chick Embryo 1146
10·2. The Nucleoplasmatic Ratio 1150
10·3. Nuclein Synthesis in Developing Eggs 1156
10·4. Creatinine, Creatine, and Guanidine 1159

Section 11. Fat Metabolism 1163
11·1. Fat Metabolism of Avian Eggs 1163
11·2. Fat Metabolism of Reptilian Eggs 1173
11·3. Fat Metabolism of Amphibian Eggs 1173
11·4. Fat Metabolism of Selachian Eggs 1180
11·5. Fat Metabolism of Teleostean Eggs 1183
11·6. Fat Metabolism of Mollusc, Worm, and Echinoderm Eggs 1184
11·7. Fat Metabolism of Insect Eggs 1184
11·8. Combustion and Synthesis of Fatty Acids in Relation to Metabolic Water 1186
11·9. Fat Metabolism of Mammalian Embryos 1190

Section 12. The Metabolism of Lipoids, Sterols, Cycloses, Phosphorus and Sulphur 1198
12·1. Phosphorus Metabolism of the Avian Egg 1198
12·2. Tissue Phosphorus Coefficients 1211
12·3. Choline in Avian Development 1215
12·4. The Metabolism of Sterols during Avian Development 1218
12·5. The Relation between Lipoids and Sterols; the Lipocytic Coefficient 1226
12·6. Cycloses and Alcohols in Avian Development 1228
12·7. Sulphur Metabolism of the Avian Egg 1232
12·8. Phosphorus, Sulphur, Choline, and Cholesterol in Reptile Eggs 1236

Section 12·9. Lipoids and Sterols in Amphibian Eggs *page* 1237
12·10. Lipoids, Sterols, and Cycloses in Fish Eggs 1239
12·11. Phosphorus, Lipoids and Sterols in Arthropod Eggs 1241
12·12. Phosphorus, Lipoids, and Sterols in Worm and Echinoderm Eggs 1243
12·13. Lipoids and Sterols in Mammalian Development 1252

VOLUME III

Section 13. Inorganic Metabolism 1255
13·1. Changes in the Distribution of Ash during Avian Development 1255
13·2. Calcium Metabolism of the Avian Egg 1260
13·3. Inorganic Metabolism of other Eggs 1268
13·4. The Absorption of Ash from Sea-water by Marine Eggs 1271
13·5. The Anion/Cation Ratio 1274
13·6. Inorganic Metabolism of Mammalian Embryos 1277
13·7. Calcium Metabolism of Mammalian Embryos 1285

Section 14. Enzymes in Ontogenesis 1289
14·1. Introduction 1289
14·2. Enzymes in Arthropod Eggs 1290
14·3. Enzymes in Mollusc, Worm, and Echinoderm Eggs 1293
14·4. Enzymes in Fish Eggs 1295
14·5. Enzymes in Amphibian Eggs 1300
14·6. Enzymes in Sauropsid Eggs 1303
14·7. Changes in Enzymic Activity during Development 1307
14·8. Enzymes of the Embryonic Body 1310
14·9. Enzymes in Mammalian Embryos 1312
14·10. The Genesis of Nucleases 1326
14·11. Foetal Autolysis 1329

Section 15. Hormones in Ontogenesis 1335
15·1. Introduction 1335
15·2. Adrenalin 1337
15·3. Insulin 1342
15·4. The Parathyroid Hormone 1346
15·5. The Hormones of the Pituitary 1346
15·6. Secretin 1348
15·7. Thyroxin 1348
15·8. Oestrin and other Sex Hormones 1353

Section 16. Vitamins in Ontogenesis *page* 1359
16·1. Vitamin A 1359
16·2. Vitamin B 1360
16·3. Vitamin C 1360
16·4. Vitamin D 1360
16·5. Vitamins in Mammalian Development 1363
16·6. Vitamin E 1365

Section 17. Pigments in Ontogenesis 1368
17·1. The Formation of Blood Pigments 1368
17·2. The Formation of Bile Pigments 1372
17·3. The Formation of Tissue Pigments 1375
17·4. The Pigments of the Avian Egg-shell 1376
17·5. The Pigments of the Avian Yolk 1378
17·6. Egg-pigments of Aquatic Animals 1380
17·7. Melanins in Ontogenesis 1381

Section 18. Resistance and Susceptibility in Embryonic Life 1383
18·1. Introduction 1383
18·2. Standard Mortality Curves 1383
18·3. Resistance to Mechanical Injury 1385
18·4. Resistance to Thermal Injury 1388
18·5. Resistance to Electrical Injury 1392
18·6. Resistance to Injury caused by Abnormal *p*H 1397
18·7. Resistance to Injury caused by Abnormal Gas Concentrations (non-Avian Embryos) 1399
18·8. Critical Points in Development 1409
18·9. Resistance to Injury caused by Abnormal Gas Concentrations (Avian Embryos) 1414
18·10. Resistance to Injury caused by Toxic Substances 1420
18·11. Resistance to Injury caused by X-rays, Radium Emanation, and Ultra-violet Light 1431

Section 19. Serology and Immunology in Embryonic Life 1444
19·1. Antigenic Properties of Eggs and Embryos 1444
19·2. The Formation of Natural Antibodies 1446
19·3. The Natural Immunity of Egg-white 1447
19·4. Inheritance of Immunity in Oviparous Animals 1451
19·5. Serology and Pregnancy 1452
19·6. Resistance of the Avian Embryo to Foreign Neoplasms 1454

| | | |
|---|---|---|
| Section 20. | Biochemistry of the Placenta | *page* 1456 |
| 20·1. | Introduction | 1456 |
| 20·2. | General Metabolism of the Placenta | 1458 |
| 20·3. | Placental Respiration | 1461 |
| 20·4. | Nitrogen Metabolism of the Placenta | 1462 |
| 20·5. | Carbohydrate Metabolism of the Placenta | 1469 |
| 20·6. | Fat and Lipoid Metabolism of the Placenta | 1472 |
| 20·7. | Placental Enzymes | 1481 |
| Section 21. | Biochemistry of the Placental Barrier | 1485 |
| 21·1. | The Autonomy of the Foetal Blood | 1485 |
| 21·2. | Evolution of the Placenta | 1487 |
| 21·3. | Histotrophe and Haemotrophe | 1492 |
| 21·4. | Mesonephros and Placenta | 1493 |
| 21·5. | Colostrum and Placenta | 1497 |
| 21·6. | Placental Transmission and Molecular Size | 1497 |
| 21·7. | Qualitative Experiments on Placental Permeability | 1505 |
| 21·8. | The Passage of Hormones | 1511 |
| 21·9. | Factors Governing Placental Transmission | 1512 |
| 21·10. | Quantitative Experiments on the Passage of Nitrogenous Substances | 1514 |
| 21·11. | Quantitative Experiments on the Passage of Phosphorus, Fats, and Sterols | 1520 |
| 21·12. | Quantitative Experiments on the Passage of Carbohydrates | 1525 |
| 21·13. | Quantitative Experiments on the Passage of Ash | 1527 |
| 21·14. | The Passage of Enzymes | 1529 |
| 21·15. | The Unequal Balance of Blood Constituents | 1530 |
| Section 22. | Biochemistry of the Amniotic and Allantoic Liquids | 1534 |
| 22·1. | Introduction | 1534 |
| 22·2. | Evolution of the Liquids | 1535 |
| 22·3. | Avian Amniotic and Allantoic Liquids | 1537 |
| 22·4. | Amount and Composition of Mammalian Amniotic and Allantoic Liquids | 1539 |
| 22·5. | Maternal Transudation and Foetal Secretion | 1546 |
| 22·6. | Interchange between Amniotic and Allantoic Liquids | 1562 |
| 22·7. | *Vernix Caseosa* | 1564 |
| Section 23. | Blood and Tissue Chemistry of the Embryo | 1565 |
| 23·1. | Blood | 1565 |
| 23·2. | Lung | 1571 |
| 23·3. | Muscle | 1574 |

Section 23·4. Heart *page* 1577
23·5. Nervous Tissue 1583
23·6. Connective Tissue 1592
23·7. Lymph 1593
23·8. Sense Organs 1594
23·9. Intestinal Tract 1594

**Section 24. Hatching and Birth** 1595
24·1. Introduction 1595
24·2. Hatching Enzymes 1595
24·3. Osmotic Hatching 1600
24·4. Egg-breakers 1602
24·5. Hatching of the Avian Egg 1602
24·6. Mammalian Birth 1605

## Epilegomena

The Two Problems of Embryology 1613
The Cleidoic Egg and its Evolution 1613
Chemical Synthesis as an Aspect of Ontogeny 1623
Biochemistry and Morphogenesis 1624
Transitory Functions in Embryonic Life 1627
The Theory of Recapitulation 1629
Recapitulation and Substitution 1632
Chemical Recapitulation 1638
Provisional Generalisations for Chemical Embryology 1647
The Organisation of Development and the Development of Organisation 1659
The Future of Embryology 1664

PART IV

## Appendices

i. Normal Tables of Magnitudes in Embryonic Growth 1669
ii. A Chemical Account of the Maturation of the Egg-cell 1679
iii. The Chemical Changes during the Metamorphosis of Insects (by Dorothy Needham) 1685
iv. The Development of the Plant Embryo from a Physico-chemical Viewpoint (by Muriel Robinson) 1711

PART V

Bibliography and Author-Index 1725
Subject-Index 1971
Index Animalium 2013

# PLATES

## VOLUME I


William Harvey . . . . . *frontispiece*

I. Primitive methods of incubation: (A) Egyptian, (B) Chinese *facing page* 46

II. The oldest known drawing of the Uterus (9th century) . „ „ 82

III. Illustration from the *Liber Scivias* of St Hildegard (*ca.* 1150 A.D.) . . . . . „ „ 96

IV. A page from Leonardo da Vinci's Anatomical Notebooks (*ca.* 1490 A.D.) . . . . . „ „ 108

V. Illustration from the *De Formatione Ovi et Pulli* of Fabricius (1604) . . . . . „ „ 116

VI. Illustration from Highmore's *History of Generation* (1651) . „ „ 134

VII. Illustrations from Malpighi: *De Ovo incubato* (1672) . „ „ 168

VIII. Réaumur's Illustration of his Incubators (1749) . . „ „ 198

IX. Microphotograph of the yolk of the hen's egg at the time of laying, to show the vitelline globules . . . . „ „ 236

X. Microphotograph of the yolk of the hen's egg, not yet liberated from the ovary, to show the stratification . . „ „ 288


## VOLUME II


The frontispiece of William Harvey's Generation of Animals (1651); Zeus liberating living beings from an egg . *frontispiece*

XI. Microphotograph of the yolk of the hen's egg at the eleventh day of incubation, showing its heterogeneous state . . . . . *facing page* 836

XII. Microphotograph of the yolk of the hen's egg at the second day of incubation, showing the cholesterol esters . . „ „ 1218


## VOLUME III


An embryological investigation in the eighteenth century *frontispiece*

# TABLES


27. Ash of the avian egg . . . . . *facing page* 302

34. Distribution of amino-acids in egg-proteins . . „ „ 330

47. Ash content of egg . . . . . „ „ 356

195. Enzymes in the hen's egg . . . . . „ „ 1304

199. Enzymes in the human embryo . . . . „ „ 1314

201. Enzymes in the pig embryo . . . . . „ „ 1316

220. Placental enzymes . . . . . „ „ 1469

227. Passage of substances through the placenta . . „ „ 1506

Appendix 1, Table 3. Embryonic growth of the hen . . „ „ 1670

## SECTION 4

## THE RESPIRATION AND HEAT-PRODUCTION OF THE EMBRYO[1]

### 4·1. Early Work on Embryonic Respiration

Probably the earliest examination of the respiration of embryos, apart from mere opinions such as those of Fabricius ab Aquapendente, was contained in the work of Spallanzani, who found that eggs gave off and took in gases, although Robert Boyle in his *Continuation of New Experiments physicomechanical touching the Spring and Weight of the Aire & their Effects* had written in 1632, "I put Flies' Egs into an empty receiver: no wormes were produc'd out of them". Nothing more of importance was done till Coxe on May 19, 1794, presented to the Academy of Sciences in Philadelphia *An inaugural Essay on Inflammation*, in which he stated that "the portion of air which we always find in one end of the hen's egg is oxygenous gas". His essay is now rare, so I have not been able to ascertain whether he regarded the contents of the air-space as pure oxygen or as simply containing pure oxygen among its constituents. Coxe thought that the air-space was of great importance for the proper growth of the embryo. Two years later, Hehl at Tubingen carried out similar work, using Fontana's modification of Priestley's eudiometer. He concluded that the air was the same as ordinary air.

In 1811 Paris made an examination of the physiology of the fowl's egg, which he communicated to the Linnean Society in London. According to his analyses, the air-space of unincubated eggs contained "pure atmospherical air", but after a development of three weeks there was "an inquination with carbonic acid". For his time, Paris held very advanced views about gaseous exchange in animals—"Is it not probable", he said, "that the repeated suspirations of the fatigued are instinctive exertions to procure a greater proportion of oxygen by which their muscular energy may be revived?" The egg-white, in Paris' view, was merely a defence against the cold. Then in 1822 Sir Everard Home made similar investigations, in the course of which he submerged eggs in water and other liquids, and observed that they would not develop, a result which he attributed to the

[1] Note: 1 mgm. $CO_2 = 0{\cdot}51$ c.c.; 1 mgm. $O_2 = 0{\cdot}70$ c.c. (at N.T.P.).

absence of the air. It is most interesting to note that none of these early workers seemed to find any difficulty in the notion of a non-pulmonary non-circulatory respiration. In 1840 Bucknell commented on the increase in size of the air-space during incubation.

Most of the attention given to embryonic respiration during the earlier half of the last century was centred on the bird's egg, but a few experiments were done on other eggs. In 1846 the Academy of Sciences in Paris offered a prize for a memoir in which the candidates were required to "determine by the aid of precise experiments what is the succession of chemical, physical, and organic changes which take place in the egg during the course of the development of the foetus in birds and batrachians". The prize was won by Baudrimont & Martin de St Ange, who produced a work which must be regarded as one of the classics of chemical embryology. I shall refer later to their general results on the metabolic changes which they investigated; it is only necessary to note here that they proved that carbon dioxide was given off throughout incubation by the developing eggs of hens, garden snails, lizards, snakes and frogs. They also measured the daily loss in weight of developing hen's eggs, and did not fail to note that this could be at least doubled by incubating the eggs in an atmosphere which had been dried by sulphuric acid. On the other hand, they affirmed that nitrogen was lost by the eggs, and that an egg weighing 50 gm. would give off 0·055 gm. of sulphur in 21 days, presumably in the form of hydrogen sulphide. They tried incubating eggs in oxygen, hydrogen and carbon dioxide, observing in each case the teratological results, and analysing the gases in the air-space. Frog's eggs placed in a vacuum were found not to have developed at all. Other points investigated by these workers were the permeability of the frog's egg to strychnine and to morphine. But, for the present purpose, it is only to be noted that they initiated the quantitative work on embryonic respiration.

Curious experiments were also made at about the same time by Rusconi and by Preyer, in which larval amphibia were raised in the absence of atmospheric air, simply by the dissolved oxygen in the circulating water, but they are not now of importance. More interesting was the gasometric work of Bischof and of Dulk, who in 1823 and 1830 respectively, without knowing of Coxe's work, analysed the gas which could be extracted from the whole egg when placed in deaerated water in a vacuum. They both found it to have a higher

oxygen tension than ordinary air. Von Baer, who was at that time writing the introductory parts of his immortal book on the embryology of the chick, was at once interested, for, just as Haldane a century later was to welcome the secretory theory of the pulmonary epithelium as a basis for views of a vitalistic character, so von Baer saw in the analyses of Bischof and Dulk a like support for his general opinions. On p. 37 he considered the gases of the egg, and, referring to a figure of 27 per cent. of oxygen (atmospheric air 20·5–21·0), said, "This value was Dr Dulk's. Previously Dr Bischof paid attention to the air in the egg and found amounts of from 22 to 25 per cent. Although his paper was rather short, I hope Dr Dulk will repeat his observations. The result of this research is for embryology and the whole of physiology so important that I feel it my duty to make very well known these valuable communications". Nothing, however, has been done on the problem since that time. But in 1847 Baudrimont & Martin de St Ange examined the air in the air-space, though it must be admitted that their analytical figures showed as often as not less oxygen in the air-space, not more, than in atmospheric air.

The most surprising feature of the early work, however, was the fact that a number of workers seriously suggested that the hen's egg was quite independent of air during its development. The main upholders of this doctrine were Erman; Viborg and Towne. Réaumur in the eighteenth century had already found (see p. 198) that development would not go on if the egg was covered with some substance impermeable to air, but Erman and Viborg, using gypsum, got different results and affirmed that air was quite unnecessary, for they could hatch out chicks from eggs buried in this way. Towne came to the same conclusion from experiments in which he gummed pieces of paper all over the outside of the shell. Von Baer, as one of his footnotes shows, was prepared to accept a good deal of this work, for it fitted in with his own idea that there was some essential difference between pre-natal and post-natal development. He anticipated, it would almost seem, the discovery of two quite different sorts of metabolism. In 1834, however, Theodor Schwann, destined to be known later on as the first cytologist, proved clearly that air was essential for development, by maintaining eggs in an atmosphere of hydrogen. His inaugural thesis at Berlin, *De necessitate aeris atmosphaerici ad evolutionem pulli in ovo incubato*, finally settled the question, though for some time other workers, such as Marshall, thought it

worth while to make further experiments (e.g. submersion in oil) in support of Schwann's conclusions[1].

A good deal of work, however, continued to be done on the effects produced by partial and total varnishing of the egg-shell, and attempts were made to find out just how much of the surface was necessary for satisfactory development. Geoffroy de St Hilaire; Baudrimont & Martin de St Ange; Herholdt; Poselger, and Dareste used varnish, collodion and wax respectively for this purpose, but, as they themselves owned, such eggs lost a good deal of weight during incubation, so that their varnishes must have been permeable to some extent to gases. This explains why in their experiments the allantoic vessels grew quite as usual under the shell. Dareste made a great many observations on these points, but he was not very successful in clearing them up. He stated that, if the part of the shell which covered the air-space was varnished, the allantois would grow over the inner parts of the shell, but not over the membrane separating the egg-contents from the air-space. If the blunt end was varnished before the fifth day, the embryos inevitably died, but if after that time, they did not, for the allantoic vessels had then had time to apply themselves to other parts of the egg's surface. Varnishing the pointed end never had any ill effects. The idea thus grew up (without any real justification) that the air-space had some special significance for embryonic respiration, or at least, that the allantois normally reached the air-space membrane first of all and so made use of its air to a special extent. Dareste also affirmed that, after the varnishing of the obtuse end, the air-space often moved round to the side of the egg. Dusing, who made a thorough examination of the whole subject at Preyer's instigation, was not able to agree with all the conclusions of Dareste. He used an asphaltic preparation which really was impermeable to gases, and carefully ascertained that the eggs varnished all over with it lost hardly any weight during their 3 weeks' incubation. By this means he found that the varnishing of the blunt end of the egg did not lead to a high mortality among the embryos, no matter when it was done, from which he concluded that the allantois does not normally reach the air-space membrane first of all, and that the embryo does not depend upon the air there for the oxygenation of the blood in its vessels. He was able

[1] Asphyxiation of insect embryos while still in their eggs has now become a very important part of economic entomology (see Staniland, Tutin & Wilson).

to hatch out a large number of eggs which had been varnished over the air-space in this way. He could not confirm Dareste's statement that the air-space could move round to one side after varnishing.

Dusing then proceeded to ascertain exactly how much of the shell surface was requisite for full oxygenation of the embryo. By varnishing little squares in chessboard formation of exactly equal area all over the egg, he found that certainly 50 per cent. of the shell could be occluded without any ill results being produced. In one case, perfectly normal development followed the occlusion of 65 to 70 per cent. of the total surface in this way, but the number of abnormal embryos rose rather rapidly at this point. Gerlach & Koch went even further, and varnished eggs over the entire surface save for a small circle from 4 to 6 mm. in diameter as near as they could judge immediately over the germinal disc. The embryos produced in eggs so treated were often abnormal, but seemed occasionally to be well developed; in all cases, however, they were much smaller and lighter than the normal. The nearer the "Luftfleck" was to the embryo, the more normal the development. Preyer drew from these experiments the conclusion that a proper supply of oxygen must be more essential for growth than for differentiation, but he did not follow out that interesting line of thought, which has affinities with Demoor's finding that irrespirable gases stop cytoplasmic streaming in *Tradescantia*, but not nuclear division (see also p. 542). Gerlach, who studied in morphological detail the embryos resulting from this method, found that the abnormalities must in many cases have arisen during the first 15 hours of development, a fact which demonstrated that, even in those early stages, oxygen was necessary. Varnishing experiments were afterwards continued by Féré and by Mitrophanov and low-pressure work by Giacomini. For the complicated early history of the work on the respiration of eggs, with all its details, Dareste's 1861 paper should be consulted.

Baudrimont & Martin de St Ange made one experiment of much interest, in view of the later work of Riddle (see Section 18·9), in which they maintained eggs at 37° in an atmosphere of 85 per cent. oxygen. They found on opening the eggs after some days that the embryo had a red colour, the allantois was a millimetre thick and very resistant, and the amniotic liquid was red, owing to the presence of numbers of erythrocytes in it. Pott & Preyer later repeated this experiment, and found that the description of the earlier workers

had been quite correct, only that no blood was to be seen in the amniotic liquid. Excess of oxygen is undoubtedly as deranging a condition as lack of it.

Pott & Preyer also investigated the normal behaviour of the air-space during development, studying its gradual enlargement and its position. They analysed the air in the air-space and naturally found carbon dioxide to be present. This latter point was contested by Berthelot, on the basis of very poor technique, so it was not surprising that Hüfner later was able to agree in full with the work of Pott & Preyer. Preyer made an attempt to explain the early figures of Dulk, etc. by Graham's atmolysis laws, thinking that the shell might be more permeable to oxygen than to nitrogen. But this matter was taken up by Hüfner, whose paper has already been referred to (p. 264), who showed that, on the contrary, nitrogen diffuses through the egg-shell more easily than oxygen.

Quantitative estimations on the loss of weight from the egg during its incubation were made very early by Sacc; Prévost & Dumas; Pfeil; Robinet (on the silkworm) and later by Prévost & Morin. "The diminution in weight", said these latter authors, "which the egg undergoes during the course of incubation, cannot be explained as a simple evaporation. It must be admitted that at the same time as the fatty substances are assimilated or destroyed, a part of the azotic bodies are too, and that there goes on in the egg an act perhaps analogous to respiration, the result of which is the exhalation of such substances as can take on the gaseous condition. The appearance of the membrane which carpets the shell seems to confirm this opinion." The simple fact of loss of weight had been known already for some time; thus Réaumur in the eighteenth century had observed a loss of 16 per cent., Copineau 14 per cent., Chevreul 17 per cent., Prout 16 per cent., Sacc 17 per cent. Falck was probably the first to make any measurements of the rate of weight loss each day, but he did not publish many weighings, and Pott & Preyer made a definite advance by increasing the number of eggs under investigation and by paying greater attention to details, such as maintenance of accurately controlled temperature and humidity. Nevertheless, their tables show great variations, and their work is not sufficiently satisfactory to be included in any calculations of importance at the present time. It is true, however, to say that the main features of the gaseous exchange of the egg were correctly

sketched out by Pott & Preyer. Thus they found that the fertilised egg developing with the embryo inside it lost on an average 19·6 per cent. of its weight, and that, if the egg was infertile and was yet incubated, it lost nearly as much (18·5), the difference being about 1 per cent. This coincides with the fact now definitely known that the main source of weight loss in incubating eggs is the evaporation of water, not more than 2 or 3 gm. of solid being burned away. The earliest observer to note that the weight loss of fertile and infertile eggs was much the same appears to have been Erman, who announced it in 1810 in a letter to Oken. Pott & Preyer also made the correct observation that the loss of weight during the incubation period was constant for each day. This, of course, made it obvious that the weight loss was not directly connected with the embryonic growth, the course of which was known by them to be curvilinear. Fig. 105 is a modification of the illustration they gave of their findings.

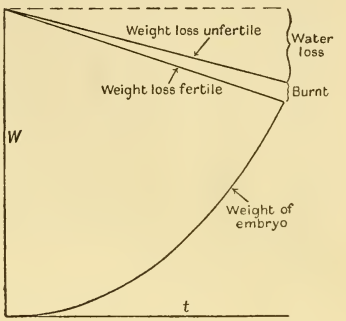

Fig. 105.

The weight of water $W$, they said, evaporated each day by the egg as far as the end of the second week, is equal to the total loss of weight, $G$, for during this time the weight of carbon dioxide produced, $K$, is exactly equivalent to the weight of oxygen absorbed, $S$—other gases being omitted on account of their small quantity. Thus

$$G = K + W - S \text{ and } G = W \text{ if } K = S.$$

Roughly speaking, these relationships are still true.

The question of whether any other gases were given off or taken in during the incubation period by the egg was also handled by Pott & Preyer. Schwann had found that not only carbon dioxide, but also hydrogen and nitrogen, were given off, a result which neither Baumgärtner nor Pott & Preyer could confirm. The work which was done later on this point, and which proved that carbon dioxide is the only gas evolved by the developing egg, will be referred to presently.

"The amount of carbonic acid gas", said Pott & Preyer, "given off by a developing embryo in a six-hour period, was four times as great at the beginning of the third week as it had been at the beginning of the second week, and on the twentieth day it was nearly ten times as great as at the end of the first week. During the course of the second week the carbonic acid gas exhaled is more than doubled and during the course of the third week it is more than doubled again." Pott & Preyer rightly felt it to be a very important finding that the embryo used up oxygen and gave off carbon dioxide long before the establishment of a pulmonary mechanism. This work would seem to be of importance in the history of the progress of the conception of tissue respiration, but no one has yet accorded such a credit to it. The absolute values which Pott & Preyer obtained for the amounts of oxygen and carbon dioxide concerned, resemble fairly closely the figures of later workers. By performing a simple calculation

$$[G_e - (K_e + W_e)] - [G_u - (K_u + W_u)],$$

where $K_e$ and $K_u$ is the loss of carbon dioxide by fertile and infertile eggs respectively, $W_e$ and $W_u$ the loss of water by fertile and infertile eggs respectively, and $G_e$ and $G_u$ the loss of total weight by fertile and infertile eggs respectively, Pott & Preyer calculated the amounts of oxygen actually used up by the embryo each day. They did not make any direct estimations of oxygen. One of their conclusions was not supported by later experiments, for they said that the loss of water was markedly affected by the presence of the embryo, affirming that fertile eggs lost much less per day than infertile ones. This statement cannot now be accepted without modification.

Although the greater part of our knowledge of the respiration of the mammalian embryo is derived from researches undertaken at a comparatively early period in the last century, the consideration of it will be deferred until the discussion of that subject. It need only be said here that Girtanner and Schehl in 1794 seem to have been the first workers to say definitely that the mammalian foetus "asphyxiates itself if it does not receive oxygen from the blood of the mother". Experiments by Scheel in 1798 led to the same conclusion. Runge & Schmidtt and Zweifel observed the presence of oxyhaemoglobin in the umbilical vessels.

Before proceeding to the investigations of more recent times, we

may mention a few more of the older ones which have a special interest. Audouin; Baumgärtner; and Prévost & Dumas all concluded that about three litres of carbon dioxide were lost during the incubation period by one hen's egg of approximately 50 gm. It is striking that, although Audouin's work, for example, was done in 1827, their value should have been so correct, for Bohr & Hasselbalch 72 years later obtained a figure of 3·032 litres. It is also interesting that the values which Pott and Nessler obtained for weight loss come very close to those of twentieth-century workers.

Early work on heat-production was, of course, much rarer. Bärensprung in 1851 ascertained by the use of a thermometer that hen's eggs in course of development were one-tenth of a degree hotter than the circumambient air, and Ruffini made a similar observation on toad's eggs. Murray had claimed as early as 1826 that the albumen at the blunt end was a degree or two hotter than that at the pointed end. In 1872 Moitessier compared the rate of cooling of fertile and infertile hen's eggs and found that the latter cooled more irregularly, perhaps because of the allantoic circulation.

## 4·2. Respiration of Echinoderm Embryos in General

Among the eggs of aquatic animals those of echinoderms have been much investigated with regard to their respiration and heat production, and they have a good deal to tell us about the gaseous exchange of the embryo. The first paper on the subject was that of Lyon, who stated that he had observed a rhythmically increasing and decreasing production of carbon dioxide during cleavage stages in *Arbacia* eggs, but as he published no figures in support of this affirmation his paper did not provoke much interest. The first work of importance was done by Warburg in 1908. Using the eggs of *Arbacia pustulosa*, he determined the oxygen consumed in a given time and at different temperatures by the Winkler method. Instead of weighing the eggs, he introduced the method of doing Kjeldahl nitrogen estimations on them, and of referring the oxygen consumed per hour to these figures, instead of to weight, on the assumption that the total protein content of the eggs remains constant through all the early stages. This was subsequently justified by Rapkine, who measured the wet and dry weights of sea-urchin's eggs. Warburg paid careful attention in this work to the possible errors

introduced by bacteria, spermatozoa, and teratological complications. His principal figures were as follows:

| | Mgm. oxygen used per hour by weight of egg corresponding to 28 mgm. nitrogen |
|---|---|
| Unfertilised egg ... ... ... | 0·055 |
| Fertilised egg ... ... ... | 0·303 |
| 8-cell stage ... ... ... | 0·355 |
| 32-cell stage ... ... ... | 0·576 |

Warburg mentioned the fact that Loeb had found that unfertilised echinoderm eggs would live well for a week in sterilised water. During this period they would absorb oxygen, so evidently a definite metabolic turnover was proceeding in them. Fertilisation led to a multiplication of the metabolic rate by 6 or 7. Later development seemed to show no change at the 8-cell stage, but some increase at the 32-cell stage.

Warburg also investigated the effect of hindering or stopping altogether the cleavage of the eggs by placing them in hypertonic sea water (1 gm. NaCl added to 100 c.c. sea water). The respiratory rate was practically unaffected, being 0·368 mgm. oxygen per hour per 28 mgm. nitrogen in the normal case and 0·347 mgm. in the inhibited case. A comparison between the rate of respiration of the egg and the spermatozoon demonstrated that the former respired about 500 times as much as the latter. The influence of hypertonic sea water, however, was found by Warburg to be a little different in the unfertilised egg. Recently Runnström has observed an increase of respiratory rate in hypertonic solutions. Loeb had shown that hypertonic solutions were active agents only if they contained dissolved oxygen, and had concluded that they had an effect on the respiratory rate. In Warburg's experiments this actually turned out to be the case; for example:

| Treatment for half hour gm. sodium chloride per 100 c.c. sea water | Mgms. oxygen used per hour per 28 mgm. nitrogen after the eggs had been put back in ordinary water |
|---|---|
| 1 | 0·085 |
| 2·3 + 1·6 c.c. *N*/10 soda | 0·282 |
| 4·3 + 3·0 c.c. *N*/10 soda | 0·535 |

Just the same effects were observed on treatment with hypotonic sea water, so it seemed as if some, at any rate, of the agents which would induce artificial parthenogenesis would also induce the high respiratory rate characteristic of the newly fertilised egg. As was to be expected, the temperature coefficient of the respiration was found to be approximately 2, for at 20° the respiratory rate of unfertilised eggs was 0·059 mg. oxygen, and at 28° it was 0·118 mg. oxygen.

In his second paper, Warburg dealt principally with the effect of

hypertonic sea water on eggs unfertilised and fertilised. He had found in the previous paper that, although the respiratory rate was practically unaffected when cleavage was stopped by putting the eggs in hypertonic sea water, they took up much more oxygen than normally on being returned to ordinary sea water. Thus treatment for varying periods with hypertonic sea water would raise the respiratory rate by a good deal. The rate of unfertilised normal eggs being taken as 1, then the rate of fertilised normal eggs was 6 to 7, that of unfertilised eggs after hypertonic sea water 4 to 5 and that of fertilised eggs after hypertonic sea water something like 20. The figures were as follows:

| | C.c. oxygen used per hr. per 28 mgm. nitrogen |
|---|---|
| Unfertilised eggs: | |
| After 75 min. in hypertonic solution 2·3 % sodium chloride | 0·29 |
| ,, 105 ,, ,, 2·3 ,, | 0·32 |
| ,, 60 ,, ,, 2·3 ,, | 0·22 |
| Fertilised eggs in ordinary sea water | 0·30 |
| Fertilised eggs: | |
| After 30 min. in hypertonic solution 4·3 % sodium chloride | 1·90 |
| ,, 60 ,, ,, 2·3 ,, | 0·71 |

It was noticeable that the utilisation of oxygen by the fertilised eggs in hypertonic solution was not equal to that by the fertilised eggs in sea water plus that by the unfertilised eggs in hypertonic solution, but was much greater. The oxygen consumption seemed to remain at a steady level, and not to rise with time, but it was very little affected by temperature, unlike the respiratory rate of normal eggs in normal sea water. More interesting embryologically was the experiment in which Warburg took eggs at different times after fertilisation, and, placing them in hypertonic sea water in order to raise their respiratory rate and get bigger differences, afterwards determined the amount of oxygen consumed by them per hour per 28 mgm. nitrogen. The figures were as follows:

| Minutes after fertilisation | Respiratory rate c.c. oxygen per hour per 28 mgm. nitrogen |
|---|---|
| 5 | 0·42 |
| 15 | 0·73 |
| 17 | 0·62 |
| 45 | 1·07 |
| 65 | 1·21 |
| 125 | 1·36 |

Time of first cleavage 100 minutes.

Here was a definite appearance of rising metabolic rate.

In his third paper Warburg took the eggs of *Strongylocentrotus* as his material. By using phenylurethane he showed that the processes of cytoplasmic and nuclear division were not very closely bound up

with the respiratory rate, for they might be greatly depressed, and even abolished altogether, without any effect on the respiration-rate being perceptible. Thus, after 25 minutes in ordinary sea water, the astropheres are visible, but nothing has happened at all in 1/2000 normal phenylurethane. After 40 minutes, the beginning of the first cleavage is usually present, but in the phenylurethane eggs the astropheres are only just appearing. After 90 minutes, the two blastomeres are usually each beginning to divide, but the phenylurethane eggs have only got as far as the equatorial plate stage. Yet in spite of these profound differences, the oxygen consumption of the two groups of eggs is not markedly different, the inhibition due to the phenylurethane not being more than 20 per cent., as opposed to the 600 per cent. rise on fertilisation. Typical figures were 0·450 c.c. oxygen per hour per 28 mgm. nitrogen for the normal ones and 0·438 c.c. for the urethane ones. "The visible changes in the early developing egg", as Warburg said, "are not conditions of the change in oxygen utilisation after fertilisation. But on the other hand Loeb discovered that oxygenation is a condition of the visible changes, so that those chemical processes, the activity of which we can judge by the amount of oxygen taken in, would seem to underly the morphological ones."

The work of Runnström on echinoderm eggs is also very important. He investigated the inhibition of respiration in mixtures of carbon monoxide and oxygen, and found that it was always greater in the case of fertilised or otherwise stimulated eggs than in the case of unfertilised ones. With 96 per cent. carbon monoxide the inhibition was on an average 64 per cent. if the egg was fertilised, but only 5 per cent. if the egg was unfertilised. He linked up these views in a theoretical discussion with the colloidal changes known to take place on fertilisation. In carbon monoxide atmospheres, membrane formation is unimpaired but no rise of respiratory rate takes place. As for potassium cyanide, very much the same results were found as for carbon monoxide, i.e. the inhibition was greatest on the fertilised eggs.

Runnström concluded from these facts that the "Atmungsferment" of Warburg (the indophenol oxidase system) is not "saturated", i.e. not fully in contact with its substrates in the unfertilised eggs. Addition of the Rohmann-Spitzer reagents makes the unfertilised eggs respire just as fast as the fertilised ones, and the inhibition by CO is then the same on both; therefore the "Atmungsferment" is just as active in the former as in the latter. Again, methylene blue is reduced anaero-

bically just as rapidly by the unfertilised eggs as by the fertilised ones, therefore the dehydrase systems are equally active in both. "Not the Atmungsferment, but the relation between Atmungsferment and its substrates, is what is changed on fertilisation" (Runnström). For a further discussion of these facts and their significance see p. 867 and the reviews of Keilin and of Dixon.

Runnström also studied the effect of urethane. Here the inhibition of the respiration of the fertilised eggs was accompanied by a stimulation of the respiration of the unfertilised ones, so that the two came to about the same level. Runnström found spontaneously occurring instances of inhibited cleavage in which the respiration was normal. He noted that the protoplasm of urethane eggs was much more heterogeneous colloidally than that of ordinary ones.

An influence which was found to be much more certain in its action and easier to control than hypertonic sea water was found to be the hydrogen ion concentration of the sea water. Herbst and Loeb had found simultaneously that this factor exercised an important influence on the normal development of the echinoderm egg, and Warburg now studied its influence on the embryonic respiration. The results were as follows:

| *p*H | Respiratory rate c.c. oxygen, etc. | Cleavage |
|---|---|---|
| 6·0 | 0·14 | None |
| 8·0 | 0·39 | Normal |
| 11·0 | 0·81 | None |

The more alkaline the sea water the larger the respiratory rate, but not necessarily accompanied by normal development. The influence of the hydrogen ion on the metabolic rate in the echinoderm embryo was evidently, therefore, not exerted indirectly through the effect on morphological development. After the experiments, the eggs were put back in normal sea water, but the number which reached the larval stage was not great either in the case of the acid ones or the alkaline ones. The effect of *p*H on the respiratory rate of *Arbacia* eggs was afterwards fully studied by Loeb & Wasteneys, and by McClendon & Mitchell.

The question of how the hydrogen ion concentration brought about this effect was also discussed by Warburg. He showed, by staining eggs vitally with neutral red after the manner originally introduced by Loeb, that the internal *p*H was not affected by a stay in sea water of abnormal *p*H. Later experiments by my wife and

myself using micro-injection methods (see p. 845) confirmed this observation of Warburg's, and showed that echinoderm eggs could remain for 3 hours in sea water at *p*H 6·0 (normal 8·4) without undergoing any change in the intracellular *p*H.

Runnström in 1929 re-opened the question of the influence of *p*H on echinoderm egg respiration. Like Warburg, he found that the more acid the *p*H the less intense the respiration; thus at *p*H 6·5 the inhibition was 27 per cent. and at *p*H 6·1 51 to 58 per cent. This effect was equally shown by fertilised and unfertilised eggs. Runnström remarked that the difference between the *p*H of the cell-interior (6·6, Needham & Needham) and that of the sea (8·4) appears to be necessary for normal respiration.

Another powerful agent which influences the respiration of these eggs is methylene blue, according to the work of Barron. Addition of this dye to the vessels in which the eggs are respiring much increases the rate at which they do so. The effect is shown, indeed, by any reversibly oxidisable dye, and Barron & Hoffmann have studied the action of the *r*H indicators (see p. 866) on egg-respiration. It depends (*a*) on the *r*H of the dye, (*b*) on the permeability of the cell. If the dye is positive to the cell the effect is maximal, and decreases with increasing negativity, for in order to bring about a raised respiration the rate of reduction of the dye must exceed the normal rate of oxygen-consumption of the cell. Here the dye is acting as an additional "Atmungsferment" and probably oxidising cytochrome. With methylene blue, the effect is not found, according to Runnström, in the case of fertilised eggs.

As a result of his experiments Warburg suggested that the toxicity of various salts for echinoderm eggs was due to their effect upon the oxidations going on in them. Thus he found that the poisonous action of sodium chloride solutions could be abolished by adding a trace of sodium cyanide to them, so that an agent which prevented the great rise in respiratory rate acted as a detoxicant. Other examples of neutralised effects could be obtained with calcium, magnesium and potassium chlorides, etc., where it was found that the nearer to normal the respiratory rate was kept the less poisonous the solutions were, and the higher percentage of normal developing embryos occurred. From the same standpoint, the influence of extremely small quantities of gold, silver and copper ions was studied. These might raise the respiratory rate by as much as 63 per cent. But their

toxic effects were annulled by traces of potassium cyanide, so that, if the increase in respiratory rate was prevented, development would go on normally after the removal of the operating substances. The rise in oxidation intensity produced by ions of the heavy metals was naturally very interesting, in view of the fact that, not long before, traces of silver had been found by Herbst and traces of copper by Delage to be parthenogenetic agents.

Parthenogenetic agents fall into five groups:

1. Hypertonic solutions (Loeb).
2. Hydroxyl ions (Loeb).
3. Traces of heavy metals (Herbst; Delage).
4. Fat-soluble acids (Loeb).
5. Fat-soluble substances such as alcohol, benzene, etc. (Hertwig; Herbst; Loeb).

Of these the first three were now found by Warburg to have a strongly stimulatory action on the respiratory rate of the fertilised eggs, while the last two had the opposite effect. Loeb had indeed found that, for the last two, the presence of oxygen was unessential, and that they would produce their parthenogenetic effect anaerobically. "The first group", said Warburg, "act primarily on the oxidations, from which follows a change in the cell-membrane and hence a change in metabolic rate. The second group act primarily on the membrane and not on oxidations." These researches of Warburg led to a long series of papers by various authors, arising out of the antagonism between sodium chloride and cyanide in affecting the respiration intensity of echinoderm eggs. Loeb & Wasteneys in 1910 went into the subject, and concluded with Warburg that the respiratory rate of eggs stimulated by salt solution was depressed by the cyanide, so that the toxic action of the sodium chloride was averted. But they disputed many of his other statements, and a polemic followed, which must be read in the original, and cannot here be discussed, as it was largely concerned with matters of technique. During the course of it many new facts came to light about the general effects of salt action on protoplasm. Loeb found that the toxic effects of many agents (neutral salts, sugars, hypertonic and hypotonic solutions, chloral hydrate, phenylurethane, chloroform and alcohol) on the fertilised *Arbacia* egg could be prevented by agents such as cyanide, which depressed the intracellular oxidations. That this was not

simply due to the suppression of cleavage was apparent from the fact that some of the toxic agents themselves (such as chloralhydrate) depressed cleavage. These questions were dealt with for some further time in the papers of Warburg; Loeb & Wasteneys and Meyerhof. Other workers who have studied the effect of various salts on the respiration of echinoderm eggs are Loucks & de Graff.

More important embryologically were the observations in which Warburg showed that not only phenylurethane would dissociate the respiratory from the morphological process, but also many other agents, such as narcosis with ammonia. The oxidative intensity, as judged by the intake of oxygen, remained unchanged although morphological development might stand quite still. Moreover, the respiratory rate could be raised to very high levels through the action of hydroxyl ions and traces of heavy metals without any morphological development going on at all. Cyanide here took a special position for, penetrating into the cell, it decreased the respiration-rate considerably, and also decreased the development-rate, not producing any deformations, but simply slowing down the normal processes.

| | Respiratory rate c.c. oxygen per hour per 28 mgm. nitrogen | Cleavage |
|---|---|---|
| In sea water ... ... ... | 0·372 | Normal |
| In *N*/100,000 sodium cyanide ... | 0·120 | Very slow |
| In *N*/10,000 ,, ... | 0·072 | None at all |

Cyanide and temperature were thus the only two agents found by Warburg which equally affected respiratory rate and morphological development.

That the increase in oxygen consumption did not rise parallel with the increase in nuclear material was fully confirmed in Warburg's third paper. The respiration-rate for *Strongylocentrotus lividus* eggs in the 2-cell stage was 0·438 c.c. oxygen, and in the 64-cell stage 0·612, an increase of only about 1·5 instead of 32 times. A few experiments in which the carbon dioxide output was estimated gave quite similar results; thus in one experiment the respiratory rate for oxygen was 0·20 c.c. of oxygen and 0·18 c.c. of carbon dioxide. Not enough work on these lines was done, however, to lead to any determinations of respiratory quotient. Possible respiratory differences between monospermic and polyspermic eggs were investigated by Warburg, and found to be slight—thus the respiratory

rate in one case for monospermic eggs was 0·456 c.c. oxygen and for polyspermic eggs was 0·498, a negligible difference.

Warburg had been led to speculate on the part played by the actual structure of the cells in metabolism by the fact that various agents, especially *p*H, seemed to affect the respiratory intensity by acting simply on the surface of the cells. He investigated, therefore, the respiration of acetone preparations of staphylococci and yeast cells, contributing the results in a joint paper with Meyerhof, who investigated the eggs of *Echinus miliaris*. The oxygen consumption of unfertilised eggs pounded up with sand was compared with that of eggs normal and untreated. In the case of unfertilised eggs, the oxygen consumption was not abolished altogether by this pulverisation, although it was definitely decreased. From 0·5 to 1·5 hours after the treatment a half to two-thirds of the original respiratory rate was found, but during the third hour this sank to between a quarter and a third of the original value. Thus the intact eggs would have a rate of 0·053 c.c. oxygen and the pulverised ones a rate of 0·033 c.c. In the case of the fertilised eggs, this fall after pulverisation was rather more pronounced; as an example:

| | Respiratory rate c.c. oxygen per hour per 28 mgm. nitrogen | |
|---|---|---|
| | Intact eggs | Pulverised eggs |
| Unfertilised ... ... ... | 0·41 | 0·23 |
| 2-cell stage ... ... ... | 0·46 | 0·14 |
| 4-cell stage ... ... ... | 0·93 | 0·11 |
| 16-cell stage ... ... ... | 0·89 | 0·26 |

But insufficient experiments were done with the fertilised eggs to enable this point to be presented with certainty. These, it may be noted, were the first respiration experiments in which manometric methods were used as opposed to the Winkler titration which had previously been universal. The acetone preparations were found to behave rather like the egg-*Breis*, for, although the oxygen consumption was much less than in the intact normal eggs, it was yet by no means absent.

The integrity of the cells was evidently only partially responsible for the oxygen uptake of the original material[1]. In a later paper Warburg pursued the question still further. He invented a method of cytolysis in which echinoderm eggs were centrifuged rapidly and shaken violently—egg-preparations according to this method not only took up oxygen, but also gave off carbon dioxide. With this

[1] Cf. Penrose & Quastel's experiments with bacteria.

material he showed that the egg-*Breis* of unfertilised eggs respired rather more strongly than the intact unfertilised eggs themselves, but that the reverse relation held true of fertilised eggs. What was very important was the demonstration that, though there was a difference of 500 to 700 per cent. between the respiratory rates of fertilised and unfertilised eggs, this difference practically disappeared in the cytolysed centrifuged material, for it possessed apparently the same respiratory rate, no matter whether it came from unfertilised or from fertilised eggs. Sometimes there might be as much as 15 per cent. in favour of the fertilised eggs, but no more. Moreover, if it was true that the spermatozoon influenced the oxidative activity of the egg almost entirely through its effect on the cell-boundary, it should follow that the addition of spermatozoa either intact or cytolysed to the egg-*Breis* would produce no rise in respiratory rate. This was actually found. Some typical figures on which the above conclusions were founded are given here.

| | Respiratory rate (c.mm. oxygen used in 20 min. by amount of egg corresponding to 20 mgm. nitrogen at 22°; several experiments) | | | | |
|---|---|---|---|---|---|
| Unfertilised eggs, intact ... | 14 | | 13 | | 12 |
| Unfertilised eggs, destroyed ... | 21 | | 22 | | 21 |
| Unfertilised eggs, destroyed ... | 25 | 22 | 26 | 23 | 21 |
| Fertilised eggs, destroyed ... | 21 | 23 | 25 | 22 | 19 |

| | Oxygen used up in c.mm. | | |
|---|---|---|---|
| Minutes | Control *Brei* alone | *Brei* plus sperm in sea water | *Brei* plus sperm in distilled water |
| 20 | 29 | 27 | 30 |
| 40 | 51 | 50 | 51 |
| 60 | 67 | 67 | 71 |

In some of the experiments Warburg estimated the carbon dioxide production, getting respiratory quotients of 0·68 and 0·80, but he laid no stress on them for various reasons connected with technique.

Warburg used these "Granulasuspensionen" of the centrifuged cytolysed eggs for the purpose of studying the effect of added iron on the oxygen uptake. It may suffice to say here that he showed that the addition of minimal amounts of iron salts increased greatly the uptake of oxygen by the centrifuged cells. He also showed that it stimulated the production of carbon dioxide by the same material, and that the excess respiration brought about by the addition of iron was of the

same type as the normal respiration, judged by behaviour towards ethyl urethane. He tried the effect of adding acids of various kinds and other substances to the *Breis*, and the inhibiting effect of the cyanide ion was cleared up on the supposition that iron-catalysed oxidations were the main ones taking place in the eggs.

About this time Loeb & Wasteneys undertook an examination of the temperature coefficient of embryonic development in the sea-urchin's egg as related to the temperature coefficient of its respiratory rate. This investigation has already been referred to in the section on growth (p. 525). In another paper they showed that fertilisation in *Arbacia* eggs led to a three to four times rise in respiratory rate, thus confirming Warburg's work on *Strongylocentrotus*.

Warburg did not omit to study the effect of varying oxygen tensions on the respiration of his echinoderm eggs. This question, a part only of a very general perplexity which has confused physiologists for many years, he answered by the finding that respiration was relatively unaffected by changes in partial pressure of oxygen. In his 1908 paper, he said, "The oxygen-concentration was so arranged that it did not sink to below $\frac{3}{4}$ of its original value, but I found that even if it sank to $\frac{1}{4}$, absorption proceeded quite regularly". Two years later he said, "I have shown that the rate of oxidation in the egg is independent of the oxygen pressure, i.e. the oxygen concentration in the egg, although this oxidation-rate can be markedly influenced by alterations in various external conditions". Warburg's figures were as follows:

| Exp. | % of oxygen | Utilisation |
|---|---|---|
| 1 | 30 | 6·6 |
| | 17 | 6·1 |
| 2 | 47 | 12·5 |
| | 25 | 12·1 |
| 3 | 33 | 4·5 |
| | 14 | 4·0 |
| 4 | 46 | 9·8 |
| | 25 | 9·2 |

Later, however, Henze went into the matter in a research which included work with anemones, gephyrean worms, and many other marine animals. His conclusion was that Warburg's sea-urchin eggs had not been well enough shaken, for a typical result of his own was

| % of oxygen | Utilisation |
|---|---|
| 37·4 | 3·7 |
| 14·2 | 2·1 |

Henze admitted that after a certain point, as was shown later for *Dixippus morosus* by v. Buddenbrock & v. Rohr, and for *Fundulus heteroclitus* by Amberson, Mayerson & Scott, the oxygen utilisation ceased to follow oxygen partial pressure. Subsequent work with other eggs has tended to confirm Henze rather than Warburg; thus Dakin & Dakin and Burfield have shown for the plaice's egg and Parnas & Krasinska for the frog's egg that the respiratory rate depends directly on the partial pressure of oxygen, below a certain point. Recently Drastich, working with the eggs of *Strongylocentrotus lividus*, has shown that there is a linear relation between cubic millimetres oxygen used

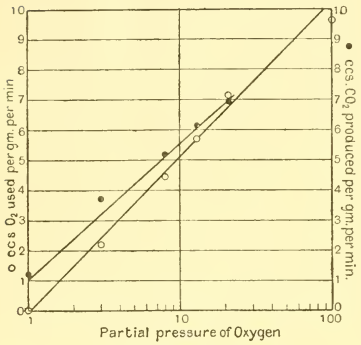


Fig. 106.

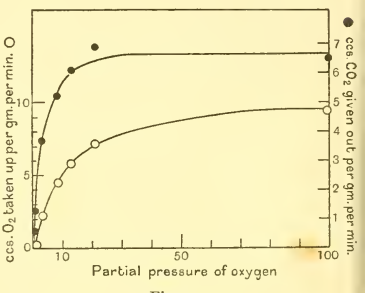


Fig. 107.

per gram per hour and log. partial pressure. This would imply a state of affairs in which the curve of oxygen consumption fell off rapidly at low partial pressures (see Figs. 106 and 107). And Amberson finds no change in oxygen consumption of *Arbacia* eggs between 228 and 20 mm. partial pressure of oxygen, though below that it falls off quickly. Everything seems to depend upon the level of oxygen concentration at which this point comes; thus Loeb, in his work on *Ctenolabrus* eggs, could find no difference in rate of development or morphology between eggs in air or in 100 per cent. oxygen, and Krogh & Johansen successfully hatched plaice eggs in oxygen pressures of one-quarter the normal (230 mm. abs.). Below this point abnormalities occurred.

Warburg returned to the subject of normal respiration in 1915,

using manometric methods instead of the Winkler titration. He first obtained a more accurate figure for the oxygen utilisation of spermatozoa, namely, 66 c.mm. oxygen per 20 mgm. nitrogen in 20 minutes at 23°. On the other hand, 20 mgm. egg nitrogen used 10–14 c.mm. oxygen in 20 minutes at 23°. Warburg found that the rise occurring at fertilisation took place within 10 minutes, during which time the

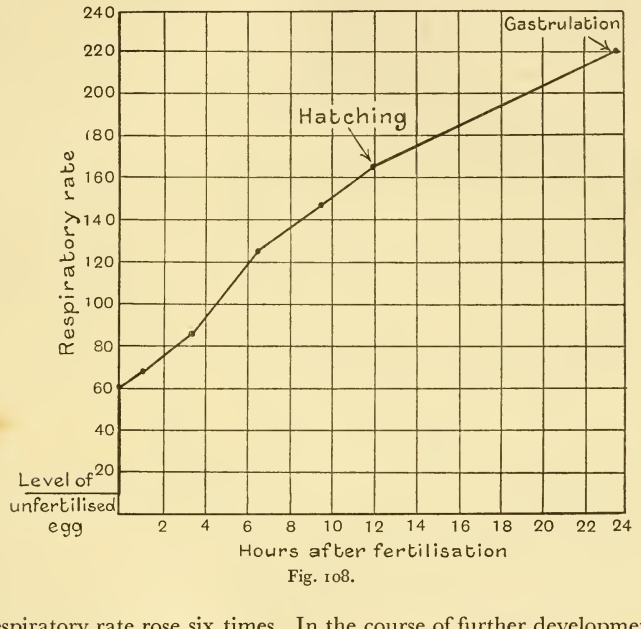


Fig. 108.

respiratory rate rose six times. In the course of further development it continued to rise, so that, after 6 hours, it had reached twelve times the value of the unfertilised egg, after 12 hours sixteen times, and after 24 hours twenty-two times. Warburg's curve, which is reproduced in Fig. 108, is very regular, and shows no peaks or rhythmic changes.

For the gaseous exchange of the fertilised *Strongylocentrotus* egg, respiratory quotients closely distributed around 0·9 were found[1].

[1] Values of unity both before and after fertilisation were later reported for echinoderm eggs by Ashbel.

Warburg did not draw any conclusion from this finding, but it has acquired importance in view of subsequent researches on the nature of the substances combusted as energy sources during embryonic development. It will be again referred to in the section on the energy relations of the growing embryo.

The figures given above demonstrated that weight for weight the spermatozoon was respiring more intensely than the egg-cell. When, however, the relations between the respiration of one spermatozoon and one egg-cell were compared, it was found that spermatozoon : unfertilised egg was as 1 : 500, while spermatozoon : fertilised egg was as 1 : 3500. Another interesting calculation which Warburg made from his experimental data was that only 0·0045 mgm. of spermatozoon nitrogen were required to fertilise 7 to 8 mgm. of egg nitrogen, i.e. to fertilise 1 mgm. of egg nitrogen $\frac{1}{1500}$ to $\frac{1}{2000}$ mgm. spermatozoon nitrogen were necessary. One conclusion from all this was that, as far as respiration experiments were concerned, it was unnecessary to make much correction for the spermatozoal respiration, owing to its extreme smallness.

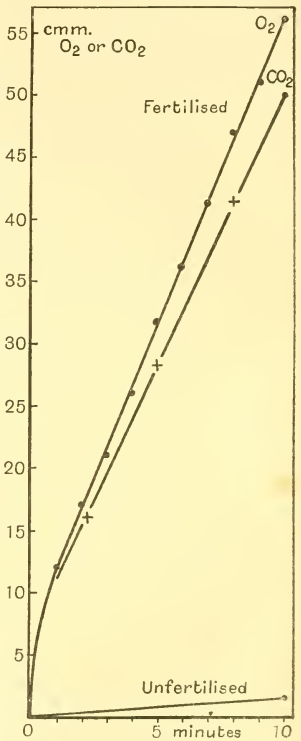

Fig. 109.

The next step forward was taken by Shearer, who in 1922, by using a special form of the Barcroft differential manometer, was able to carry out the fertilisation of *Echinus microtuberculatus* eggs actually inside the closed chamber of the apparatus, and observe more intimately still the earliest stages of the embryonic respiration.

Fig. 109, taken from Shearer's paper, shows the phenomena which may under such conditions be observed during the first 10 minutes, i.e. during the period which elapsed between fertilisation and the

first readings of Warburg's curve. The two curves show the oxygen consumed and the carbon dioxide in c.mm. given off during the fertilisation and early development of an amount of egg-substance corresponding to 4·06 mgm. of nitrogen (approximately half a million eggs) in the case of *Echinus microtuberculatus*. The respiratory quotient for this experiment was 0·92. The lower line in Fig. 109 shows the oxygen consumption of half a million unfertilised eggs (1·5 c.mm. in 10 minutes). The difference between this figure and the 56 c.mm. of oxygen taken up in the same period immediately after fertilisation is very striking. All the other graphs obtained by Shearer were of the same form, from which it is evident that the uptake of oxygen in the first minute is not only many times more than during any minute before fertilisation, but also more than at any subsequent minute. After the first couple of minutes the rate of increase of metabolic rate falls off, and the curve ascends rather less steeply. The oxygen consumption per unit weight (calculated on nitrogen basis) of the unfertilised eggs per minute was found to be 0·15 c.mm., but the same eggs fertilised consumed in the first minute after the addition of the spermatozoa 12 c.mm. of oxygen, an increase of about eighty times the former value. Shearer compared the respiratory rate of the eggs before and after fertilisation with that of the liver of a well-fed cat (from values in the literature) and obtained the following comparison:

| | Respiratory rate |
|---|---|
| Cat liver ... ... ... | 10·7 |
| Unfertilised egg ... ... | 0·37 |
| Fertilised egg ... ... ... | 13·8 |

Now examination of sections of fixed material of *Echinus* eggs during the process of fertilisation shows that the spermatozoa take at least 10 to 15 minutes to embed themselves in the cytoplasm of the egg. In material fixed within 2 or 3 minutes of the addition of spermatozoa to eggs the former are found only attached to the external surface of the egg-membrane, not having had time to penetrate it. Comparison of these facts, then, with the experimental evidence, makes it clear that the initial burst of oxygen consumption must be brought about simply by the first contact of the sperm with the outside of the egg-membrane. The great rise in metabolic rate which occurs in the very earliest stages of development cannot depend on the formation of the male pronucleus in the cytoplasm, and must be due to some activity exerted by the spermatozoon before it has entered the egg at all. Moreover, when the fusion of

the male and female pronucleus takes place later on there is no fresh rise in the process, but rather a further slowing down. This is illustrated by Fig. 110, which shows an experiment covering a longer period. At the 25-minute point, where the nuclei fuse, there is no kink in the curve, and the process is slowing off. "The nuclear features of syngamy", as Shearer puts it, "seem connected in no direct way with the oxidations taking place in the ovum."

Such a conclusion is in agreement with the work of Warburg, which, as we have already seen, led to the view that the surface of the egg was of particular importance with regard to the oxidations proceeding in it, and hence the respiratory rate. Injury to the egg-membrane is invariably followed by a great increase of oxygen consumption of echinoderm eggs, and, as will be noted presently, Meyerhof found that similar treatment was accompanied by a definite increase in heat production. In eggs treated with hypotonic sodium chloride solutions, the absence of calcium and potassium ions interferes with the normal condition of the cell-wall, and the oxygen consumption rises to five or six times the normal. In just the same way the heat production rises from 0·9 to 3·4 gm. cal. per hour after treatment with valerianic acid, which induces artificial membrane formation. There is as yet no satisfactory explanation for these phenomena, for the most recent knowledge which has accumulated about oxidation processes can hardly allow us to be satisfied with the simple conception of an accumulation of iron in the surface layer of the egg, as Warburg suggested. Even if it were there, and were released in some way by parthenogenetic agents, it does not, as is now known, catalyse all types of oxidation. However, Runnström's work has shown that the rise in metabolic rate which occurs at fertilisation is connected both with the colloidal state of the cell-interior and with the degree of contact between the "Atmungsferment" and its substrates.

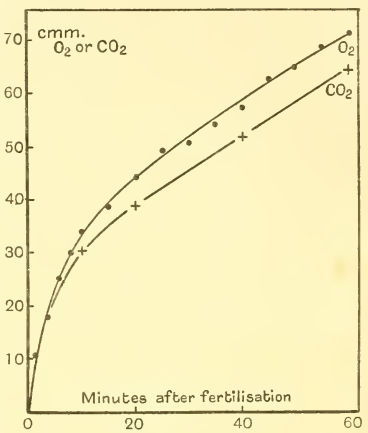


Fig. 110.

There is no need to suppose that the "Atmungsferment" is concentrated in the cortical layers of the egg, for in a protoplasmic system the transmission of a physical change would readily occur (see also p. 867).

Shearer also carried out some experiments on the glutathione content of the eggs before and after fertilisation. He stated that, before fertilisation, they only gave a weak nitro-prusside reaction, but that, immediately after fertilisation, deep magenta colours could be obtained by this test, indicating the presence of reduced glutathione in some quantity. "There seems to be fairly substantial ground for believing", he said, "that there is an immediate increase in the quantity of this remarkable body in the ovum on fertilisation." Work with the nitroprusside test, however, must be interpreted with caution until quantitative estimations of glutathione have been done on the early stages of the developing echinoderm egg by the iodine method or by some other suitable technique[1].

As regards the great initial rise in metabolic rate in the first minute of the experiment, there is every probability that it represents the results of egg-oxidations. But in view of the recent work of Gray, this cannot be said to be a certainty, for Gray, examining the respiration of spermatozoa, finds them to have a much larger and more variable metabolic rate than had been suspected by the earlier workers. Warburg's original figures relating spermatozoa oxygen consumption to egg oxygen consumption were obtained in very concentrated sperm suspensions, and Gray has been able to observe much higher rates of oxidation, especially during that period of activity which the spermatozoa go through just before the eggs are fertilised.

Other and less important measurements have been made of the oxygen consumption of echinoderm eggs before and after fertilisation. Thus McClendon & Mitchell in 1912, using the Winkler method, demonstrated a rise of six to eight times the previous value at fertilisation, whether natural or parthenogenetic, in the case of *Arbacia punctulata*. McClendon's theory was that fertilisation led to permeability changes in the eggs which permitted a greater volume of gas to pass in and out per unit time.

As will be seen later, Meyerhof made a good many estimations of oxygen consumption during the early stages of development of the sea-urchin's egg, in connection with his researches on the calorific

[1] Rapkine's more quantitative experiments indeed show a diminution of SH on fertilisation followed by a rise to the time of first cleavage.

quotient and the heat production. He did not devote much attention to analysing his oxygen data, since they were obtained only as a means to an end, but Bialascewicz & Bledovski showed later that they fell on a parabolic curve which was fitted by the equation

$$x = kt^2 + a,$$

where $x$ is the respiratory rate at time $t$, $a$ the rate in the case of the unfertilised egg, and $k$ a constant. They drew no theoretical conclusion from this, nor did they give the constant in question.

So far all the workers mentioned have been in agreement concerning the effect produced on the oxygen consumption of echinoderm eggs on fertilisation. The first discordant voice was that of Loeb & Wasteneys, who in 1912 reported that the eggs of the starfish (species not given) took up no more oxygen after fertilisation than before. The measurements were made by Winkler's method, and the number of experiments described was small, so that the paper did not attract much attention until Fauré-Fremiet's similar results with *Sabellaria*. Later still, Barron & Titelbaum found no rise in respiratory rate on fertilisation in *Nereis*. The work on the starfish has been confirmed by Barron. That there is a tremendous rise in metabolic rate, however, during the early cleavage stages of most echinoderm eggs cannot now be doubted, although the shape of the curve in the few minutes after fertilisation is still open to revision[1].

Much interest attaches to some recent experiments of Carter in which the effect of the egg-secretions upon the spermatozoa were studied. The known factors here are: (1) Lillie's effect—the agglutination of spermatozoa; (2) Glaser's effect—the lipolytic action (see Section 14·3); and (3) Gray's effect—the increase in oxygen consumption of the spermatozoa. This increase was not found by Carter to follow the same laws as Gray had stated, i.e. there was no falling off of rate (unless the sperm cells were unripe) but on the contrary a constant uptake. This constant uptake was uninfluenced by thyroxin although the falling uptake of unripe cells was increased by that hormone. As iodine is a well-known parthenogenetic agent, there are some possibilities that iodine or a thyroxin-like body is contained in egg-secretion.

[1] It is interesting to note that increased respiratory rate on fertilisation has been reported for the minnow (Boyd), the silkworm (Ashbel), the tunicate, *Ciona intestinalis* (Runnström), and various amphibians (Bialascewicz & Bledovski; Parnas & Krasinska).

## 4·3. Rhythms in Respiratory Exchange

From the researches we have so far been discussing no evidence has been found of a rhythmical activity in the gaseous exchange of echinoderm eggs. Lyon's paper of 1904 has been referred to, however. "In nearly all experiments", he said, "there was an increase in $CO_2$ production during the first ten or fifteen minute interval following fertilisation. The increase was slight, and sometimes could not be detected. Following this came an interval in which the $CO_2$ production was small, visibly less indeed in two or three experiments than that of the unfertilised eggs and sperm. This is the mid-period of cleavage, approximating perhaps to the time of nuclear growth and the early stages of karyokinesis. The interval during which the eggs were dividing into the first two blastomeres was one of active $CO_2$ production. After this period came an interval of lessened production. In one or two cases a second rise occurred at about the time of the second cleavage." And in his second paper on respiration and susceptibility, he said, "It may be stated that the apparent conclusion was that $CO_2$ production in the egg is not uniform throughout the whole series of morphological changes in cell-division, but rather reaches a maximum at the time when the cytoplasm is actively dividing. Furthermore it seemed that at the time when oxygen is most necessary and presumably is being used in largest amount (as indicated by susceptibility to lack of oxygen and to KCN) $CO_2$ is produced in largest amount. If the conclusion above expressed should justify itself it would indicate that oxygen is used chiefly in the egg for synthesis rather than for combustion, and that the larger part of the $CO_2$ comes from splitting processes. One would also infer that the energy for cell-division comes from fermentative rather than oxidative processes". Obviously these points were of great importance for a knowledge of the metabolism of the egg during the earliest stages of its development, and it was unfortunate that Lyon gave no figures in support of his opinions. The theories of cell-division subsequently associated with the names of Robertson, McClendon, Spek and Heidenhain would then have been easier to assess, for they all postulated the existence of some more or less actively contractile mechanism in the cell during cleavage in contradistinction to the theory of Gray, in which cleavage is essentially due to a rearrangement of the different cell-phases round the growing

asters, and in which sudden changes in surface energy of the cell at its equator are not involved. If a contractile mechanism of any sort exists in the dividing cells of the early embryo it would be reasonable to expect some sort of periodicity in the gaseous exchange, just as muscular exertion would be expected to produce it. The sudden activity of the cleaving mechanism would probably be marked by a change in the observable properties of the developing embryo. Lyon's work, then, failed to give an answer to a very important question. In 1922 Vlès made an investigation of the problem, using the eggs of *Paracentrotus lividus* and a quite new method, consisting of the immersion of the developing eggs in a solution of thymol-sulphonephthalein, and the observation of the slight colour changes in the indicator by a spectrophotometer. The method was an ingenious one, and the results obtained by it afforded evidence of a rhythm of carbon dioxide production. As the indicator measurements only gave indirect evidence of the formation of acid, a curve with amount of carbon dioxide as one axis could not be drawn, but Vlès constructed instead a curve relating *p*H to time. For about 3·5 hours after fertilisation the solution surrounding the eggs became more and more acid, but after that time the acidity rose rapidly, falling off and reaching a little plateau just before the completion of the first cleavage. During the 2-cell stage, exactly the same relationships were observed, first of all a rapid rise, and then a falling off to a plateau immediately preceding division. During the stages of 4, 8 and 16 cells, the same cycle was repeated, but the formation of the blastula stopped the rhythmical process, and the eggs then remained for some time causing only a slight change in the tint of the indicator surrounding them. Vlès concluded that blastulation involves a change of some kind in the metabolism of the embryo. He recalled in connection with these rhythms the cyclical behaviour noted by Herlant, Heilbrunn and many other workers in susceptibility, viscosity, etc., during early embryonic cleavage (see Section 18).

Gray determined to test the question experimentally by following the cell-divisions through, while estimating the oxygen consumed manometrically. This he did, paying careful attention to the necessary precautions, and obtaining the results shown in Fig. 111. The smoothness of the curve is remarkable, and gives no indication at all of any rhythms. Fig. 112, also taken from his paper, shows the result of taking the slope of the curve in Fig. 111 at successive points, and

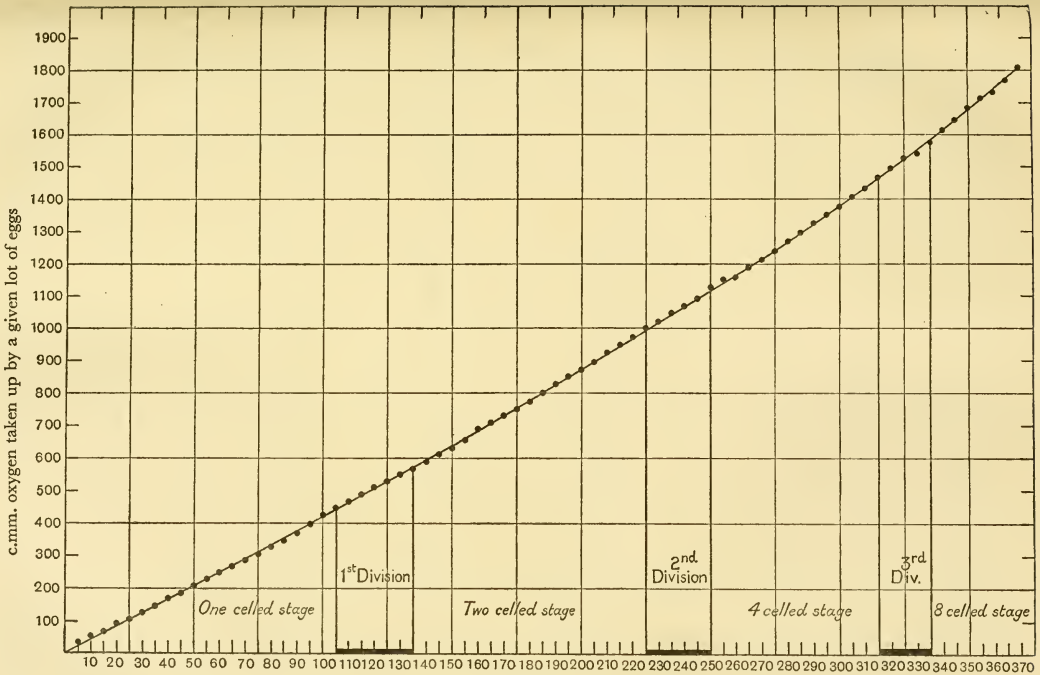
c.mm. oxygen taken up by a given lot of eggs
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370
minutes
One celled stage
1st Division
Two celled stage
2nd Division
4 celled stage
3rd Div.
8 celled stage

Fig. 111.

plotting them together. The rate rises clearly from about 21 to about 33 per cent. during the 375 minutes elapsing between fertilisation and the end of the 8-cell stage. It is obvious from this graph that the fluctuations in the rate of oxygen consumption bear no relation to the periods of cleavage, but must depend on other variable factors. The curves given by Vlès are comparable with the curve for oxygen consumption given by Gray in Fig. 111, so that there is a contradiction between the results obtained by the two methods. In such a case, one can only accept the results given by the most direct and accurate method, so that it is necessary to conclude with Gray that

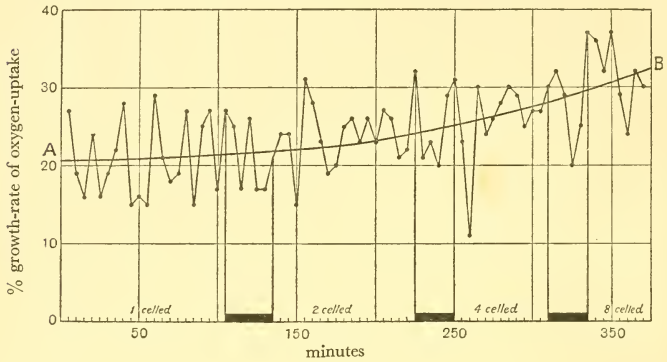


Fig. 112.

there are no rhythms in metabolic intensity during the cleavage of the echinoderm egg. For the details of the criticism of Vlès' technique the original papers must be referred to. It should be observed that Vlès' rhythms of carbon dioxide production do not agree with those of Lyon, for in the former case the period of maximum carbon dioxide evolution occurs immediately after division, and in the latter case during it. The rhythms of Lyon correspond rather to the rhythms of susceptibility to various agents which the echinoderm egg has been found to undergo during its cleavage stages. These will be discussed in the Section on susceptibility. Having decided, then, that the oxygen consumption gives a better measure of the metabolism in a case such as that of the sea-urchin's egg, there remains the possibility that the rhythms observed by Vlès in carbon dioxide evolution may be real

phenomena. For, supposing that the carbon dioxide were produced in the cell at a uniform rate just as the oxygen is apparently consumed at a uniform rate, there might still be difficulties in observing this. Nothing is yet known about the alkali reserve of the echinoderm egg, and other factors, such as the alkalinity of the external medium and the surface area of the egg, not accurately controlled in Vlès' experiments, might be expected to play a large part in conditioning the results. "It is clear", said Gray, "that since the periodicity in carbon dioxide output is not accompanied by a periodicity in oxygen uptake, the former cannot be regarded as of significance in respect to energy changes in the egg, unless they be due to some obscure form of anaerobic activity for which there is no evidence."

The smooth curve obtained by Gray for oxygen consumption of eggs is in agreement with the earlier observations of Warburg, using the Winkler method. It is a very important key position to have gained, for in the light of it various theories which have been proposed from time to time are stripped of some of their attraction. Thus Matthews and Osterhout both claimed that the nucleus played an active part in the oxidative mechanisms of the cell. This is an old idea, and we have already referred to it in connection with the "growth-catalyst". It was the basis of R. S. Lillie's work on intracellular oxidations, and the association of ideas "nucleus-oxidations-growth" lies at the bottom of much of Child's writing. Its influence can be traced through region after region of investigation between 1900 and 1918. Gray's work on the oxygen consumption of *Echinus* eggs demonstrated that, if the nucleus had anything to do with oxidations, its influence must be altogether independent of the phase of nuclear activity, and must be unaffected by the presence or absence of definite nuclear boundary. Again, Robertson in his book suggested that differentiation and growth were dependent on the relative concentration of some catalyst in the nuclear and cytoplasmic portions of cells. This autocatalyst being formed within the nucleus can only enter the cytoplasm when the nuclear membrane breaks down during each prophase. Robertson does not actually say that the autocatalyst is an oxidising agent, but he accepts the view that the presence of the substance can be gauged by the intensity of cell-respiration. On Robertson's theory, therefore, rhythmic changes during early embryonic development in the gaseous exchange would certainly be expected, and it is interesting that,

where they can be measured best, no rhythmical phenomena occur. The facts are much more in agreement with the view of Warburg, expressed in his review of 1914, that the respiration of echinoderm eggs is a function of the cytoplasm, and is independent of the synthesis of the nucleus. Matthews' observation that, in the absence of oxygen, the astral rays disappeared, need not mean that they use oxygen, but simply that, in the absence of the fundamental metabolic processes normally going on, the morphological processes are inhibited. This resembles some of the effects we have already noticed in the work of Warburg.

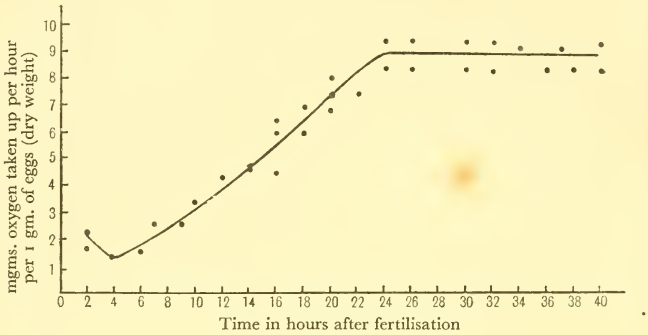


Fig. 113.

Shearer followed the oxygen consumption of echinoderm eggs for the first 10 minutes after fertilisation, Gray as far as about 16 hours, i.e. as far as the 16-cell stage in certain cases, and Warburg as far as 25 hours, i.e. gastrulation. Rapkine in 1927 followed the process further still. Warburg and Shearer referred their determinations to amounts of nitrogen, but Rapkine improved on this by using dry weight. His estimations were done by the Winkler method. The results were rather perplexing, for, when the mgms. of oxygen consumed per 1 gm. dry weight of eggs were plotted against time (see Fig. 113), a curve was obtained which ascended slowly towards the 24th hour, after which it entered on a plateau indicating apparently a uniform metabolic rate as far as the 40th hour. This in itself agrees fairly well with Warburg's curve, except for a slight initial fall about the 3rd hour after fertilisation, for which there is no

explanation. The difficulties which have already been mentioned with respect to Vlès' work on the carbon dioxide production naturally operated in Rapkine's work as well, though it was much more satisfactory in so far as the gas was bubbled off, and the carbon dioxide estimated by a baryta method. The alkali reserve, of course, remained an unknown factor. The curve for carbon dioxide given out per hour per gram dry weight followed for the first 9 hours a regular course concave to the abscissa. After that time, however (see Fig. 114), it dropped to a trough at the 15th, and afterwards rose again to a peak at the 24th, after which it again fell and rose to the 40th hour, i.e. to the pluteus stage. The large waves seen in the curve

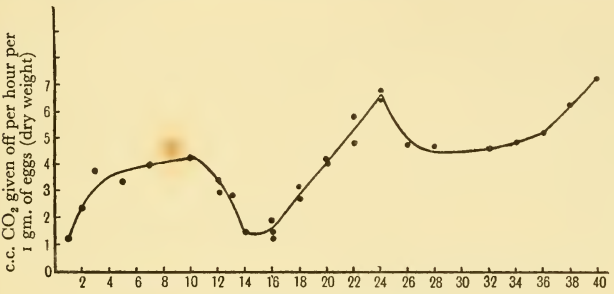


Fig. 114.

after the 10th hour are undoubtedly due to the formation of the skeleton, which is so prominent a feature of the later development of the echinoderm egg As Rapkine & Prenant showed, very definite *p*H changes take place in the blastocoele cavity as the calcareous spicules are formed and the mesenchyme develops, which are associated with the retention of carbon dioxide to form calcium and magnesium carbonates. Moreover, as Herbst showed, if potassium chloride is added to the water, skeleton formation is inhibited, and in such a case Rapkine found a regularly rising carbon dioxide curve. The respiration curves after the 10th hour are therefore difficult to interpret, and attention must rather be directed to the time elapsing before that point of development is reached. During this early period, the metabolic rate, both as regards oxygen and carbon dioxide, is rising, but significantly not at the same rate, so

that, when the respiratory quotient is calculated, it begins just after fertilisation at about unity (good agreement here with Shearer and Warburg), but then rises to the extreme value of 4·0 at 4 hours of development, after which it falls away to unity, and remains there (due allowance being made for the influence of calcification) until the pluteus stage is reached (see Fig. 115). Rapkine concluded from this that the enormously high respiratory quotient of the 4th hour indicated a period dominated by syntheses and in which a minimal amount of combustion was proceeding. Coupled oxidation-reduction reactions, the significance of which for embryonic development as a whole will be dealt with in the section on Energetics, would here

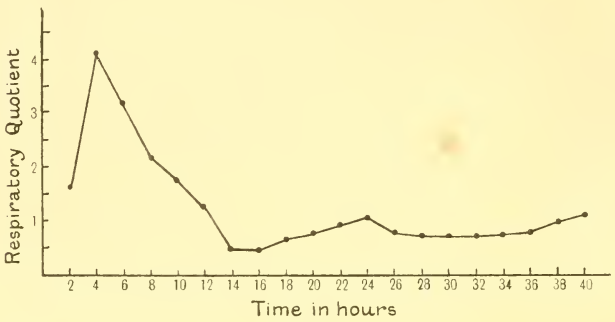


Fig. 115.

be proceeding to a greater extent than simple combustions, so that the carbon dioxide put out would be out of all ordinary relation to the oxygen taken in. Were this the case, the heat given out as actually found calorimetrically would be smaller than that calculated from the absorption of oxygen (endothermic reactions, which catch and hold the heat for the organism, predominating), unlike the state of affairs found to hold for the chick's later stages by Bohr & Hasselbalch. Now Rapkine in a later paper calculated that this was actually the case. Shearer found that, during the first 12 hours of development in the sea-urchin, 251·96 calories were given out, but the heat corresponding to complete combustion of 31·3 mgm. of protein, and 1·65 mgm. of fat (chemical analyses of Rapkine) amounted to 331·3 gm. cal. or half as much again. The discordance between

the observed and calculated values was even greater if the figures of Meyerhof for heat production were used, and somewhat less if those of Rogers & Cole were substituted for them, but in no case did the results of the two methods coincide, the difference being at least 40 gm. cal. These facts led Rapkine to the conclusion that in this early period simple combustion was to a great extent complicated by coupled reactions, one member of which was endothermic. He saw in the low calorific quotients of Meyerhof and the preliminary heat absorption phase of Bohr & Hasselbalch still further indications that such processes might be by no means negligible.

## 4·4. Heat-production and Calorific Quotients of Echinoderm Embryos

The work on the heat-production of echinoderm eggs divides itself roughly into the long papers of Meyerhof in 1911, the work of Shearer in 1922, and of Rogers & Cole in 1925. Meyerhof made use of a simple apparatus consisting of a Dewar flask immersed in an accurate thermostat together with a Beckmann thermometer, giving readings correct to ·001°. With this apparatus he made many experiments on the production of heat by the developing sea-urchin embryos. One of his typical results was as follows:

| | Heat produced in gm. cal. per hour per amount of *S rongylocentrotus lividus* eggs corresponding to 140 mgm. nitrogen |
|---|---|
| Eggs before fertilisation ... ... ... | 0·89–0·91 |
| 1st hour after fertilisation ... ... | 4·0 –4·2 |
| 2nd hour (transition to 2-cell) ... ... | 4·5 –5·0 |
| 3rd hour (4-cell stage) ... ... ... | 5·3 –5·8 |
| 4th hour (8-cell stage) ... ... ... | 6·0 –6·5 |
| 5th hour (16- to 32-cell stages) ... ... | 7·8 –9·5 |
| 6th hour (32- to 64-cell stages) ... ... | 9·8 |
| 14th hour (larvae begin to swim) ... | 12·9 |
| 18th hour ... ... ... ... | 17·8 |

Another of his experiments is shown in Fig. 116, taken from his paper. It is interesting to note that, like the curve of Gray for the oxygen consumption of echinoderm eggs, it shows no variations from its smooth course corresponding to the periods of cleavage. During the first 18 hours, the heat-production rate increases by four times, i.e. fairly parallel with the oxygen uptake rate. Membrane formation did not seem to have any effect on the heat-production, for eggs which had stood so long in sea water that they had lost the power

of raising fertilisation membranes gave exactly similar results in the calorimeter. If the fertilisation membrane, however, was artificially produced in various ways, the heat production of the eggs might be raised considerably above the normal. The spermatozoa, which were also studied by Meyerhof, had an extremely small heat-production compared to that of the eggs; thus approximately 10 milliards of sperms gave off 4·6 cal. per hour when perfectly fresh, but after 3 hours in sea water this value had fallen to 3·1 cal. per hour.

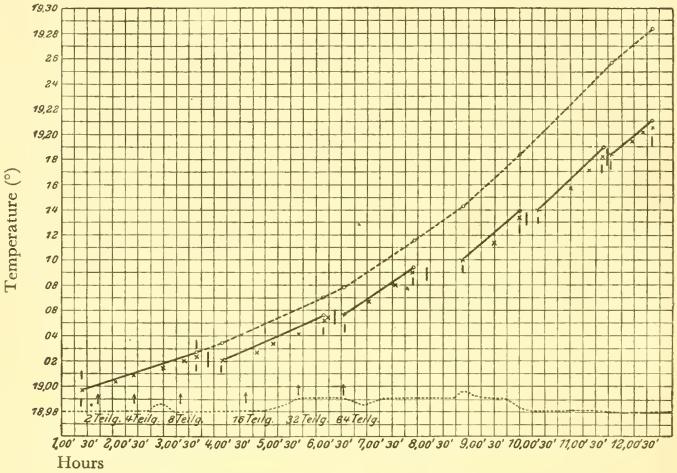


Fig. 116.

The determination of the heat-production of the eggs was in itself very interesting, but Meyerhof went further, and compared it with the oxygen uptake of the same eggs, in order to obtain the calorific quotient, i.e.

$$\frac{\text{Heat given off by 1 unit of material in gm. cal. per hour}}{\text{Oxygen taken in by 1 unit of material in mgm. per hour}}.$$

This quotient, like the respiratory quotient, gives some sort of index of the type of metabolism going on inside the living cells under investigation. It was called by Pflüger the "caloric coefficient of

oxygen", and various workers had obtained the theoretical values for it, as follows:

| | Zuntz | Rubner | Pflüger | Average |
|---|---|---|---|---|
| For protein ... ... | 3·14 | 3·0 | 3·3 –3·24 | 3·2 |
| For fat ... ... | 3·28 | 3·27 | 3·29 | 3·3 |
| For carbohydrate ... | 3·54 | — | 3·53–3·40 | 3·5 |

The differences are very small, and this probably accounts for the fact that less is heard of calorific than of respiratory quotients in the general literature. Nevertheless, in cases where the estimation of carbon dioxide output is difficult, such as eggs whose alkali reserve is unknown, the calorific quotient is very valuable. Meyerhof estimated it for normally developing eggs—leaving out of account eggs without membranes, etc.—as 2·75, 2·88, 2·675, 2·7, 2·675, 2·85, and 2·7; these were his seven quite satisfactory experiments. Roughly speaking, it wavered between 2·55 and 2·9, a value obviously much underneath the theoretical for any of the three main classes of energy source, although, as the lowest theoretical number was for protein, they lay nearer that than any of the others. These relationships are shown diagrammatically in Fig. 117. There was apparently no variation at all in the calorific quotient during development, as the following table shows:

| | Calorific quotient |
|---|---|
| Unfertilised eggs ... ... | 2·8, 2·525 |
| 1- to 2-cell stages ... ... | 2·75, 2·775, 2·675 |
| Morula stages ... ... | 2·7, 2·55 |
| Swimming larvae ... ... | 2·675 |
| Blastulae ... ... ... | 2·8 |

Some explanation was evidently required to deal with the marked lowness of all the figures. What made the situation still more perplexing was that these figures were uncorrected for the heat of solution of carbon dioxide in sea water and for other phenomena consequent upon the carbon dioxide output of the eggs. Meyerhof did not estimate the carbon dioxide directly, but calculated its effects from the few estimations which had already been done by Warburg, and, when this correction was made, the average calorific quotient sank to 2·6.

Meyerhof also found that, just as phenylurethane had been found by Warburg to inhibit cleavage while leaving respiration untouched, so it had no effect on the heat production, and, therefore, none on the calorific quotient. The calorific quotient of eggs in phenylurethane solutions was 2·65 to 2·75, a finding which conclusively

showed that no chemical energy disappeared to form morphological structures, for, had that been the case, the calorific quotient must have been different when development ceased. Again, for parthenogenetic eggs with membranes raised by valerianic acid, the corrected

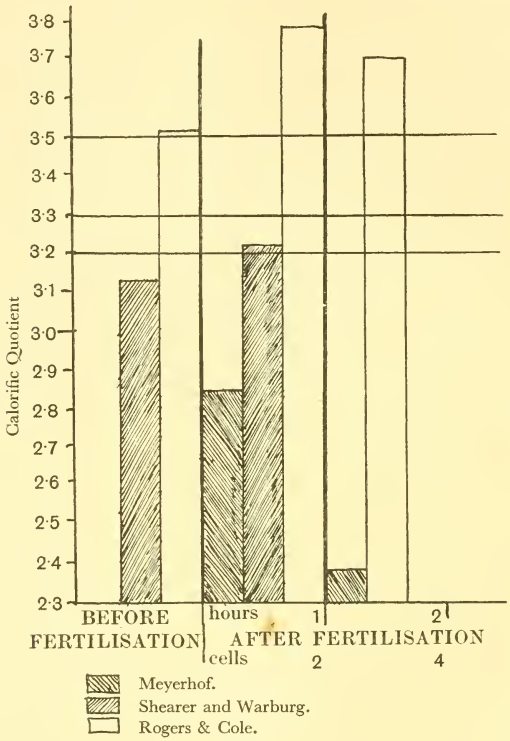


Fig. 117.

calorific quotient was 2·6. A more unexpected result was the calorific quotient of eggs in sea water to which ammonia had been added. Here, as Warburg showed, no cleavage goes on, but the respiratory rate is slightly raised. Meyerhof found that the calorific quotient of such eggs was 3·25 to 3·35, and this was the only case in which

he got values at all resembling the theoretical ones. He was not able to devise a satisfactory explanation for this. Hypertonic sea water, which had a great effect on the heat-production rate, just as it had on the respiratory rate, made very little difference to the calorific quotient. In a sodium chloride solution of 4·3 per cent. it was 2·6, in one of 3·5 per cent. it was 2·85, in one of 2·3 per cent. it was 2·9 (unfertilised) and 2·85 (fertilised). The calorific quotient of spermatozoa was found to be between 3·05 and 3·1, from which Meyerhof concluded that perhaps they were making use of protein as a source of energy.

Indeed, the question of the interpretation of the low calorific quotient was the main point of interest to Meyerhof. He examined the *Strongylocentrotus* eggs for glycogen and free glucose, and could find no trace of either[1]. Nor could he detect any nitrogenous breakdown-products in the sea water surrounding the eggs (Nessler's reagent). But by the use of the Kumagawa-Suto method, he did succeed in revealing the presence of fatty substances in the unfertilised eggs, obtaining for an amount of egg-mass equivalent to 140 mgm. nitrogen, 1·905 gm. ash-free dry substance, a total ether extract of 0·323 gm., which contained 0·282 gm. of saponifiable fatty acids. This would correspond to 14·8 per cent. of fatty acid and 2·15 per cent. of lipoids and sterols (dry weight). This material might then be used to supply the necessary energy. Meyerhof supposed that there were three possibilities: (1) That the oxygen was all being used for the oxidation of the fatty acids, but that at the same time certain strongly endothermic processes were going on which accounted for the missing 25 per cent. of the heat. In certain conditions, e.g. sea water containing ammonia, these endothermic processes would be considered to be abolished, and the full amount of heat permitted to escape, giving a reasonable calorific quotient. (2) The oxygen consumed might not all be used for the combustion of the fat, but might partly be employed in synthetic processes without accompanying heat-production. We have already met with this idea in Rapkine's work. Lastly (3), the source of energy might not be exclusively fatty acids, but other substances, burning in exothermic manner, and sharing the total oxygen. In the two last-named cases the energy-content or calorific value of the eggs would decrease during development, even if some of the products of combustion were retained inside the cells, but, in the first alternative, this would not occur. Put in another way, the

[1] But see on this, Section 8·11.

question would be, does the calorific value of the eggs decrease or increase, and, in either case, what relation does it bear to the wet and dry weights of the eggs. Evidently, the only way to ascertain what these relations were was to investigate the eggs during their development with the aid of a bomb calorimeter, and Meyerhof promised a study on these lines. But perhaps because of the great difficulties such a continuation of his work, which would have been extremely interesting, was never published.

To the three possible explanations of Meyerhof's low calorific quotients, however, a fourth might have been added, namely, the suggestion that, in spite of all his precautions, a consistent leakage of heat was going on in his apparatus. The great advances which have been made in recent years as regards the measurement of the heat-production of muscle tissue have shown how difficult it is to be sure of registering all the heat eliminated. Impressed with such considerations as these, Shearer in 1922 determined to re-investigate the matter. He criticised Meyerhof's technique on various grounds, e.g. use of too crowded cell-suspensions, insufficient aeration, reliance upon the Winkler method instead of on manometric methods. Shearer had already shown in the case of bacteria that the cytolysis of the cells was accompanied by a greatly increased oxygen intake and heat-production, so in this work he took special precautions against it.

His differential microcalorimeter was substantially the same as that elaborated by A. V. Hill for muscle, consisting of two vacuum flasks, a copper-constantan thermocouple, and a very sensitive galvanometer. Elaborate precautions were taken to prevent errors and to find the total amount of heat produced. Shearer felt that the most important result of Meyerhof's experiments was that whether he took the unfertilised egg, the fertilised egg, or the fertilised egg treated with phenylurethane, so that no cell formation was going on, although the egg was fully alive, he found that the value of the calorific quotient was always the same. Yet if any of the chemical energy liberated in the egg as the result of the increased oxygen consumption on fertilisation were utilised in producing the visible morphological structure of the embryo, the calorific quotient could not have been the same in all the instances.

Fig. 118 gives a graph showing one of Shearer's experiments. The calorimeter contained an amount of eggs corresponding to 58·4 mgm. nitrogen. In the 1st hour after fertilisation the eggs liberated

2·9 gm. cal., in the 5th hour 10·5, and in the 11th hour 22·8. All these figures, except the first one, are rather higher than the corresponding ones of Meyerhof, though it must be remembered that Shearer was working with *Echinus miliaris* and Meyerhof with *Strongylocentrotus lividus*. In another experiment where 146·2 mgm. egg nitrogen were present, 6·34 gm. cal. were given off in the 1st hour, 28·0 gm. cal. in the 5th hour, and 74·4 in the 11th hour. For the 1st hour following fertilisation Shearer obtained a calorific quotient of 3·22, while for the unfertilised egg his average figure was 3·07. He did not wish, however, to draw any conclusions from this difference, for, in view of the large numbers of eggs which had to be used in the case of eggs before fertilisation, and the inevitable individual differences between females in ripeness, physiological condition, etc., it was perhaps wise not to lay too great stress on the variation between the unfertilised and fertilised eggs. Nevertheless, the values obtained were much nearer the theoretical values than those of Meyerhof, so that it was not unlikely that these more accurate measurements had overcome the loss of heat which had led to his low calorific quotients. As Fig. 117 shows, Shearer's result worked out at about the level of protein combustion, although he himself laid no stress at all upon this fact; and certainly it did not agree very well with the respiratory quotient of 0·95 which he obtained simultaneously on the same material. What seemed to him of especial importance was that there was no marked difference between the calorific quotient of unfertilised and of fertilised eggs,

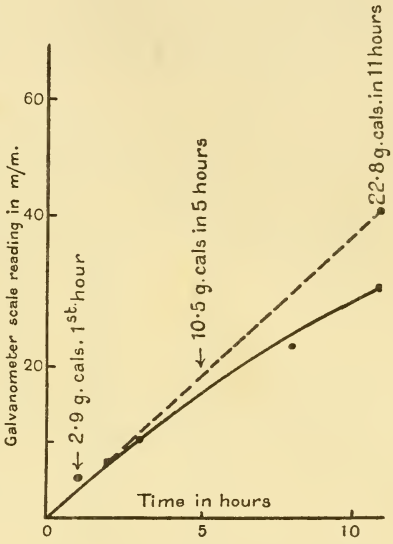


Fig. 118.

and in this he confirmed Meyerhof's findings. He therefore concluded that only a negligible quantity of the energy liberated in the high metabolic intensity of the fertilised egg-cell was expended in bringing into being the visible morphological structure of the embryo. It was employed, he thought, in keeping the living substance itself intact as a physical system. Energy used for this purpose would presumably come under the heading of developmental work or "Entwicklungsarbeit", and this problem will be fully discussed in a succeeding section, but it may be said here that all the

Table 78.

| Hours after fertilisation | Heat-production in gm. cal. per gm. dry weight per hour | | |
|---|---|---|---|
| | Meyerhof | Shearer | Rogers & Cole |
| 1 | 3·05 | 5·3 | 6·4 |
| 2 | 3·43 | 8·8 | — |
| 3 | 4·05 | 12·2 | — |
| 4 | 4·50 | 16·0 | — |
| 5 | 5·00 | 19·3 | — |
| 6 | 5·50 | 23·0 | — |
| 7 | 5·95 | 26·0 | — |
| 8 | 6·50 | 30·0 | — |
| 9 | 7·00 | 33·0 | — |
| 10 | 7·60 | 36·6 | — |
| 11 | 8·25 | 41·8 | — |
| 12 | 8·80 | — | — |
| 13 | 9·30 | — | — |
| 14 | 9·85 | — | — |
| 15 | 10·70 | — | — |
| 16 | 11·60 | — | — |
| 17 | 12·50 | — | — |
| 18 | 13·60 | — | — |

evidence goes to show that it is very small proportionately to the general energy turnover of the embryo. At the same time, it remains a remarkable fact that the calorific quotient of the early stages of echinoderm development, at any rate, should be exactly the same no matter whether morphological differentiation is proceeding or not, i.e. before fertilisation and under phenyl-urethane, as against normal development after fertilisation. All these facts together with others which have been referred to above go to show the comparative independence of processes collaborating simultaneously in embryonic development to produce the finished organism. The developmental mechanisms do not function at the same rate or with the same rhythm, and the great problem of the future is

the nature of their integration. At present we are only uncovering, as if by a kind of dissection process, the various contributory systems, such as those represented in Murray's diphasic schema for the chick.

Shearer found that in 1 hour 1 million unfertilised eggs (corresponding approximately to 8 mgm. egg nitrogen) consumed 15·1 c.mm. of oxygen and gave off at the same time 0·067 gm. cal. at standard temperature and pressure. After fertilisation the same quantity of egg-substance consumed 86·4 c.mm. of oxygen and liberated 0·3976 gm. cal. heat together with an amount of carbon dioxide equivalent to a respiratory quotient of 0·92. These figures were afterwards gone over again by Rogers & Cole, who desired to introduce even more accurate methods, and to take readings at short intervals over considerable periods of time. Rogers & Cole only published one paper, most of which was taken up with problems of technique. In Shearer's experiments, the vacuum flasks were undergoing a gradual fall of temperature throughout the experiment, and the difference in rate of fall between the control flask and the flask containing the experimental material gave the heat-production of the eggs. Rogers & Cole, however, used a different method, by which the fall was much slower, and therefore much longer experiments could be carried out. Fig. 119 shows one of their experiments. The total number of eggs used was 3·9 millions. The curve well demonstrates the absence of any variations due to cleavage. In Fig. 120 the average rate of heat-production in calories per hour per million eggs from all their experiments is given. It is probable that not

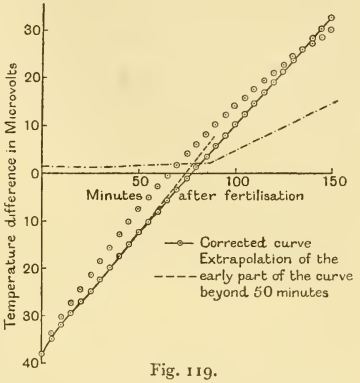


Fig. 119.

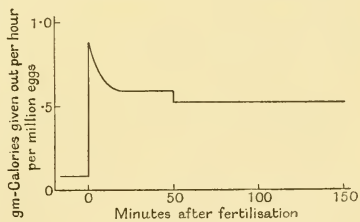


Fig. 120.

much attention should be paid to the exact shape of the curve, but only to its general form. It is very interesting that the heat-production rate should be for the first 3 hours after fertilisation a constant. This does not agree with the results of the other two workers, who found that the rate of heat-production rose during early development more or less parallel with the respiratory rate. The rate of heat-production of the unfertilised *Arbacia* egg, according to Rogers & Cole, is 0·08 gm. cal. per hour, and during the 2-cell stage 0·52 gm. cal. per hour, figures which are distinctly higher than those found by Shearer and by Meyerhof.

| | Gm. cal. heat liberated per 1 million eggs (8 mgm. egg nitrogen) per hour |
|---|---|
| Before fertilisation (Meyerhof) ... ... ... | 0·0514 |
| ,, (Shearer) ... ... ... | 0·067 |
| ,, (Rogers & Cole) ... ... | 0·08 |
| After fertilisation (2-cells) (Meyerhof) ... | 0·272 |
| ,, ,, (Shearer) ... ... | 0·40 |
| ,, ,, (Rogers & Cole) ... | 0·52 |

It is evident, however, that the percentage increase is just the same. Rogers & Cole drew no conclusions from these facts, but there is some likelihood that their figures are more accurate than those of the earlier workers, for, where slight losses of heat are the most probable cause of error, we ought perhaps to accept the highest figures as the best ones. It was obviously worth while to calculate calorific quotients for *Arbacia* again on the basis of the highest figures for heat-production, and this I did in 1927. Unfortunately Rogers & Cole never made any estimations of oxygen consumption on their eggs, and, although Loeb & Wasteneys stated in 1910 that they had made measurements of respiratory rate on *Arbacia* eggs confirming Warburg's work, these seem never to have been published, so that the calculation could not be more than an interesting feeler. Using Shearer's figure for oxygen consumption (bearing in mind that it was obtained on *Echinus*, not *Arbacia*) and Rogers & Cole's figure for heat-production, the calorific quotient for the fertilised eggs (1st hour) worked out at 3·8, not 3·215, and for fertilised eggs (2nd hour) at 3·7, not 2·37. Similarly, for the unfertilised egg, the calorific quotient worked out at 3·51, not 3·13 or 3·07. When these values are placed beside the older ones of Meyerhof and Shearer, as in Fig. 117, it can be seen that they overpass all the theoretical levels just as the earlier figures failed to reach them. There may be some significance in the fact that the

calorific quotients thus calculated approach the carbohydrate level rather than any of the others, for, as will appear later, there is much evidence associating a combustion of carbohydrate solely or predominantly with the earliest stages of development.

## 4·5. Respiration of Annelid, Nematode, Rotifer, and Molluscan Embryos

Fauré-Fremiet, in the course of his researches on the physiology of the egg of the polychaete worm, *Sabellaria alveolata*, carried out some determinations on the gaseous exchange, although, as far as I can find, his figures are not to be found in the literature in full. Using the Lévy-Marboutin technique for estimation of dissolved oxygen in sea water, Fauré-Fremiet found that the unfertilised eggs consumed almost as much oxygen as the fertilised ones. Thus 100 gm. of egg consumed in 100 minutes before fertilisation 42 mgm. of oxygen, and afterwards 47 mgm. of oxygen at 20°. At 19° the relative figures were 36 and 38, at 16° 15 and 16, at 11° 13 and 13, and at 0° 8 and 7. The feeble rise in respiratory rate (not more than 12 per cent.) appeared, therefore, to fade away as the temperature was lowered, a fact which led Fauré-Fremiet to conclude that it was not of essentially the same character as the 800 per cent. rises invariably found in the case of the fertilised echinoderm egg. From these figures he calculated a temperature coefficient which was 1·6 between 0 and 10° and 3·2 between 10 and 20°. As regards the rise in respiratory rate on fertilisation, it may, owing to loss in raising the cultures, etc., have been rather higher than the figures actually show. Fauré-Fremiet also estimated the liberation of carbon dioxide from these eggs, using a modified form of the Osterhout-Haas method, i.e. titration of sea water in which the eggs have been respiring, to different end points with various indicators. Fauré-Fremiet did not publish the figures he obtained by this method, and did not venture to calculate a respiratory quotient from them, but merely stated that "the relation between the values found is always near enough to unity to constitute a verification of the results observed, or at any rate of their order of magnitude". I do not quite understand what this means, but we may conclude that there is some evidence, at any rate, in favour of the respiratory quotient of *Sabellaria* eggs in the segmentation stages being about 1·0. The resemblance between this figure and those obtained for segmenting echinoderm eggs will be evident.

The respiration of nematode eggs has also been studied by Fauré-Fremiet, who employed *Ascaris megalocephala* as material, and the old-fashioned Bonnier-Mangin apparatus as technique. Fig. 121 shows the curves he obtained for oxygen intake and carbon dioxide output. These are for 1 gm. of dry weight, and so are true metabolic rate curves. They differ very much for those obtained on all other animals, for, instead of rising as development proceeds, as occurs both as regards respiratory rate and total amount respired in all other cases, they maintain a practically uniform level. Their absolute value approaches that of some fragmentary figures later given by Holthusen. One gram dry weight of vermiform embryos ready to hatch thus consume no more oxygen in 24 hours than one gram

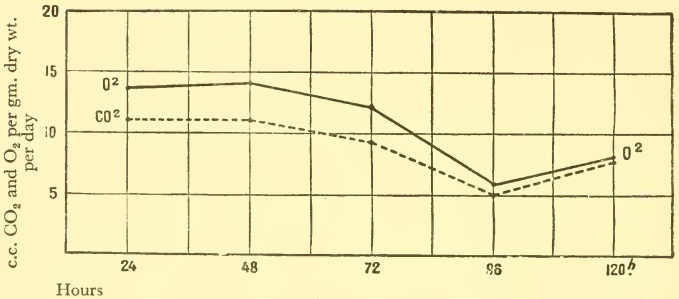


Fig. 121.

of dry weight of newly fertilised eggs, a strange state of affairs, which may be related to the fact that, in later life, *Ascaris* differs from all the other examples in being eventually anaerobic. During development, Fauré-Fremiet found that 50 c.c. of oxygen were absorbed and 43·8 c.c. of carbon dioxide given out per gram dry weight, and this led to some interesting calculations concerning the general metabolism, for which see later. The respiratory quotient for the whole period was 0·876, but, when it was calculated for each day during the 120 hours of development, the graph shown in Fig. 122 was obtained. During the earlier stages of development, the respiratory quotient fell from 0·82 at the 24th hour to reach 0·74 at the 72nd hour, after which it rose steadily, though more rapidly at first than later, to 0·92 at the end of development. It was therefore declining during the stages of segmentation and gastrulation, it

reached its minimum at the time when the embryos were curved like a U, and it rose again during the main period of increase in length and the assumption of mobility by the embryos. If, then, the *Ascaris* egg is burning its reserves completely to carbon dioxide and water, it could be stated roughly that a period of protein or mixed fat-protein combustion was succeeded by a period of carbohydrate or mixed protein-carbohydrate combustion.

Later work by Brown shows that a difference of temperature has no influence on the amount of oxygen taken up by nematode eggs during their development, but only affects the rate at which this process occurs[1]. Zavadovski has found that also in the case of the nematode egg (*Ascaris*) cleavage is stopped by lack of oxygen or by certain

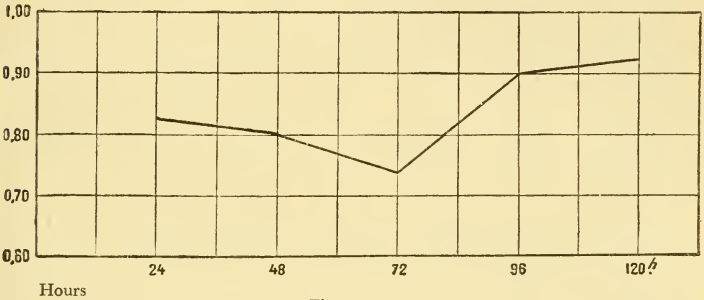


Fig. 122.

concentrations of potassium cyanide. He has brought forward evidence showing that this egg has two kinds of oxidation-processes, one group affected by potassium cyanide and the other not affected, and that cell-cleavage is associated with the former group. Rezničenko, like Zavadovski, has studied the effect of potassium cyanide (in large amounts) on the respiration of nematode eggs, but with paradoxical results.

Lite & Whitney have made some observations on the respiration of rotifer eggs. These do not normally hatch for many weeks after they have been laid, but if they are laid without a shell or with only a very thin one they develop fast, and hatch in a comparatively

[1] This was confirmed by McCoy for the eggs of the hookworm, *Ankylostoma caninum*. These are exactly the same size as *Ascaris* eggs and take up the same amount of oxygen from fertilisation to hatching, although their speed of development under the same conditions is 21 times as fast. High oxygen tensions inhibit development of hookworm eggs.

short time. Lite & Whitney found that by vigorously aerating the water in which the eggs of *Brachionus* and *Asplanchna* were lying, they could make the thick-shelled ones develop as fast as the thin-shelled ones, and hatch as soon. They concluded, therefore, that the respiratory rate is much influenced by the amount of air available and that the developmental rate follows it.

Quantitative experiments were made by Buglia on the eggs of *Aplysia limacina*, the sea-hare, a gastropod mollusc. He employed Vernon's method for estimating the gases dissolved in sea water, which involves very elaborate apparatus, and gives a fair degree of accuracy, and he estimated the oxygen taken in and carbon dioxide given off. Fig. 123 *a* taken from his paper shows the curves which he got by plotting the cubic centimetres of oxygen taken in or carbon dioxide given out per kilogram of eggs per hour against the time from laying, all at three different temperatures. It will be seen that the carbon dioxide and the oxygen run closely parallel during the greater part of the time, and that there is a very marked rise in respiratory rate between the 30th and 60th hours. It is not easy to understand why at the lowest temperature this rise should be almost abolished, unless the time taken in development was then so long that it had not begun at the 100th hour, by which time the other two curves had long attained a steady level. Nor does the upper curve (30°) agree with such a presentation of the data, for it would be expected to rise much more sharply than the curve at 20°, whereas, on the contrary, it rises more slowly. The respiratory quotients worked out as follows:

Table 79.

| Hours | State of embryo | Respiratory quotient 10° | 20° | 30° |
|---|---|---|---|---|
| 8 | 1-cell ... ... ... ... ... | — | 0·81 | — |
| 12 | 44-cell ... ... ... ... ... | — | 0·85 | — |
| 13 | Beginning of morula ... ... ... | — | 0·98 | 1·02 |
| 20 | Morula ... ... ... ... ... | — | 0·72 | — |
| 25 | First segmentations of nutritive blastomeres | 1·00 | 0·90 | — |
| 27 | Morulation of nutritive blastomeres ... | — | 0·90 | 0·88 |
| 30 | Ectoderm nearly surrounds endoderm ... | — | 1·06 | — |
| 35 | Ectoderm completely surrounds endoderm | 1·25 | 0·94 | 0·92 |
| 50 | Half-formed embryo ... ... ... | — | 1·19 | — |
| 60 | Embryo quite formed ... ... ... | 1·00 | 1·12 | 0·95 |
| 120 | Embryo with many cilia ... ... ... | 0·71 | 1·11 | — |
| 150 | Embryo with rapid rotatory movement ... | — | 0·98 | 0·95 |

If the figures for oxygen uptake and carbon dioxide production during the 2 hours of an experiment were compared, it was found

that in the early stages the eggs had a lower gaseous exchange during the 2nd hour than during the 1st, but that in the later stages the reverse was the case, and the 2nd hour readings were greater than the 1st. This was interpreted to mean a decreasing

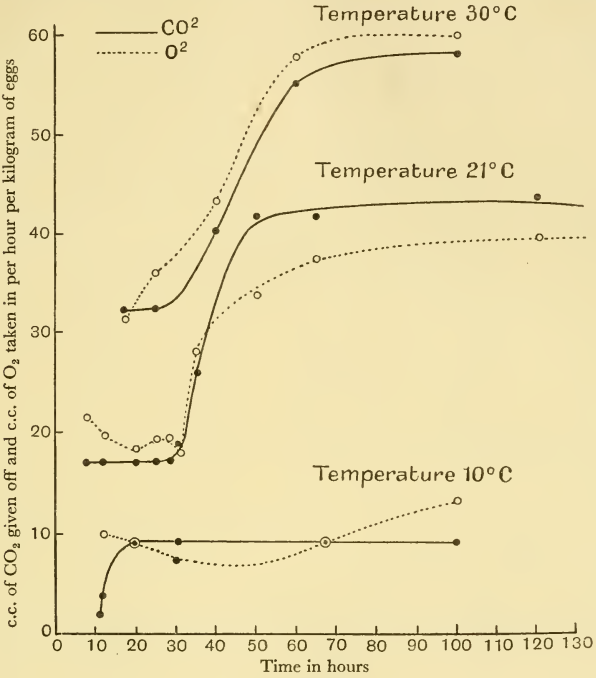


Fig. 123 *a* (Buglia).

sensitivity with age to the effects of carbon dioxide in the ambient sea water. Returning to Fig. 123 *a*, the curve at 21° should be taken as normal, and from that it is clear that there are three periods in the change of respiratory rate with time in the case of *Aplysia*, (*a*) from 0 to 35 hours, (*b*) from 35 to 50 hours and (*c*) from 50 hours onwards. The point marking the transition of period (*a*) to period (*b*) is the complete surrounding of the endoderm by the ectoderm, while

the point marking the close of the great rise is complete sketching out of the embryo with most of its parts. A study of Carazzi's monograph on the development of *Aplysia* does not reveal any more striking correlations. It is worth noting that Buglia's figures for metabolic rate must be accepted with the reservation that we do not yet know how the dry weight of the gastropod eggs varies during their development, nor how the total nitrogen behaves in relation to the total wet and dry weight. It is therefore not possible as yet to say how far Buglia's curves are comparable with those which have already been given for the metabolic rate of echinoderm eggs (i.e. related to so many mgm. of egg nitrogen). Nevertheless, it is interesting that the respiratory rate seems to increase as *Aplysia* develops, rising between two steady levels, just as that of echinoderms does. Whether these metabolic rates are at all comparable with those for the chick, for instance, is not sure, for in so many cases, e.g. amphibia and fishes, as well as this gastropod, it is either not possible or else very difficult to measure the actual amount of protoplasmic substance at any given moment, and the quantities of inert yolk must falsify the results a great deal.

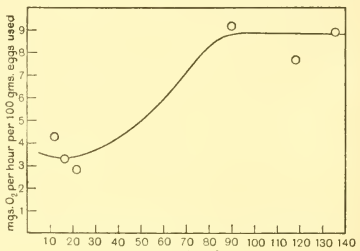

Fig. 123 *b* (Meyerhof).

Meyerhof continued Buglia's work on *Aplysia* by making some determinations of its heat-production at different stages of development. He used the same apparatus as had been employed for his heat-production work on echinoderm eggs, and did oxygen estimations by the Winkler method. The calorific quotient worked out at 2·8 for the early and 2·9 for the late stages. Meyerhof confirmed Buglia's S-shaped curve for respiratory rate in these eggs (see Fig. 123 *b*) and concluded from the calorific quotients that fat was being burned as a source of energy throughout development, pointing out the correspondence between this finding and the richness of the eggs in yolk compared with echinoderms.

## 4·6. Respiration of Fish Embryos

A good deal of work has been done on the physiological and morphological side of the respiration of fish embryos, but only little on the physico-chemical side. As an example of the first type of work, the experiments of Polimanti might be mentioned. He gave a good account of the first respiratory movements of the eggs of *Scyllium canicula*, and described their association with the development of the nervous system. Then a good deal is known about the probable respiratory function of structures in some of the rarer fishes; thus Ryder states that in the surf-perch, *Ditrema laterale*, the caudal fin has hypertrophied blood-vessels *in utero* which serve as respiratory portals for the embryo. This dermal vascularity disappears before birth[1]. Compare this with the similar structures in tropical land frog embryos described by Barbour. These questions will be further discussed in the sections on the placenta.

Quantitative observations began in 1896 with Bataillon, who measured the carbon dioxide evolved by the eggs of various kinds of teleosteans, such as the perch, the minnow (*Phoxinus laevis*), the vaudoise (*Luciscus jaculus*), the rousse (*Luciscus rutilus*) and the gudgeon. He raised his minnow embryos in a current of moist air free from carbon dioxide, and not in water, and found that their development proceeded perfectly normally in such conditions. He concluded that there were two periods in development at which the carbon dioxide given off per hour was rather low, one at a stage just preceding the extension of the blastoderm over the yolk-sac, and one after the occlusion of the blastopore. In later experiments he actually placed the eggs in weak baryta, and found that they de-

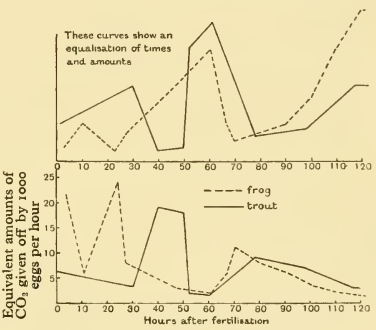

Fig. 124.

[1] A remarkably interesting adaptation occurs in the case of the lung-fish, *Lepidosiren paradoxa*. The eggs develop in burrows where the water contains no measurable dissolved oxygen (Carter & Beadle), but the male fish which guards the nest has long vascular filaments on its pelvic fins during the breeding-season, and these may secrete oxygen into the water around the eggs (Cunningham).

veloped normally, after which a titration at varying intervals was all that was necessary to give him his curve. In Fig. 124 are shown some curves constructed from the data which he obtained. They are neither smooth nor regular, but Bataillon, it must be remembered, was engaged in pioneer work. The continuous line is the curve for the minnow. The curves show, as he himself pointed out, (*a*) a rise during segmentation followed by (*b*) an accentuated fall during the extension of the blastoderm over the yolk-sac, after which the curve climbs again (*c*) to a peak at the end of the covering of the yolk by the blastoderm. Then there is a period (*d*) of lowering at the time of the occlusion of the yolk-plug, followed by (*e*) a slow rise till the beginning of active movement. It is difficult to know what emphasis to lay upon these results, for the description of the technique by which they were obtained is so short that no adequate idea can be had of it, and it presents, moreover, suspicious features.

It may be remarked in passing that the curves given by Bataillon are increment curves, and thus show up minor variations in the more or less regular curve relating time to oxygen uptake.

Scott & Kellicott in 1916 made an extended study of the respiratory exchange of the embryos of another minnow, *Fundulus heteroclitus*. Unfortunately, this was never published, and all that we have is an abstract which gives nothing but the main points, and those almost too briefly to be of service. According to Scott & Kellicott, in the early cleavage stages 1000 eggs use 0·1 c.c. of oxygen per hour. The appearance of the circulation of the blood produces a marked rise, after which another steady level supervenes, so that at hatching they are using 0·7 c.c. of oxygen per hour, having consumed throughout the entire period from fertilisation to hatching about 80 c.c. Six days after hatching they use 1·75 c.c. per hour. From fertilisation to hatching 38 per cent. of the egg-weight is lost, presumably by combustion. One thousand eggs in early cleavage stages were found by Scott & Kellicott to consist of 0·12 gm. of protoplasm and 2·65 gm. of yolk, but 6 days after hatching they weighed 1·8 gm.; 0·12 gm. of protoplasm used 0·1 c.c. of oxygen per hour, so that the metabolic rate for the early cleavage stages was 8·34 c.c. oxygen per hour per 100 gm., and from the figures given for 6 days after hatching it was 9·74 c.c.

Hyman also worked on *Fundulus* eggs with the Winkler method.

Fig. 125 shows the undulatory curve which she obtained in her experiments, with peaks at 2, 4 and 10 days after fertilisation. The values, it will be noted, are of the same order as Scott & Kellicott's. The segmentation stages were all passed through during the first upward rise of the curve, which reaches its peak at the time when the morula stages (though the term is inappropriate for a teleostean egg) have all been passed through, and the shape of the embryo

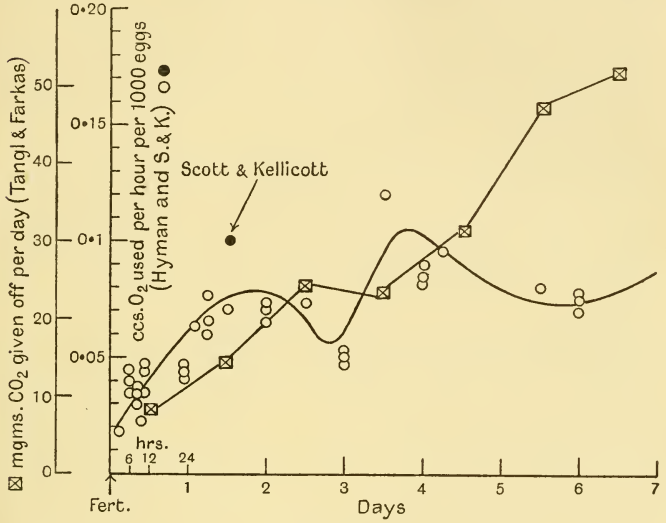


Fig. 125.

appears clearly for the first time. Just prior to this point, the germ ring has been approaching the equator of the egg, and what corresponds to gastrulation has been proceeding. After this time, there is apparently a fall to the point at which the heart begins to beat, but thereupon a rapid rise takes place, which is presumably the same as that referred to by Scott & Kellicott in connection with active circulation. Later values are described by Hyman as "irregular", but when the actual figures she gives are plotted on the same graph they only show a gradual fall, followed by a gradual rise. It is to

be feared that the number of points secured by Hyman is insufficient to establish so sharply inflected a curve. It gives us little indication concerning the metabolic rate for we do not know the rate at which the non-respiring yolk is disappearing, but Hyman did not hesitate to conclude that "the respiration is probably highest per unit weight of protoplasm early on the second day of development since from that time on the amount of protoplasm increases greatly but the oxygen consumption does not increase in like proportion, in fact, a considerable part of the oxygen consumption after the third day is due to the activity of the heart. As the embryo is continually increasing in size after this time while the oxygen consumption per hour shows little increase relatively, we may reasonably conclude that the oxygen consumption per unit weight of the embryo is actually decreasing". Hyman also made estimations of the carbon dioxide production, observing the time required for 40 eggs to take a given amount of sea water from *p*H 8·2 to 7·6, but these she did not publish, simply stating that "the study of the $CO_2$ production yielded similar results. It increased per unit time up to the early part of the second day of development after which it fell, rising again in later periods".

A comparison between the results of Hyman on the oxygen consumption of *Fundulus* and Bataillon on the carbon dioxide production of *Phonixus* is of interest. Bataillon's work was apparently unknown to Hyman, but it can hardly be mere coincidence that the general tenor of the curves should be the same. The first peak in Hyman's curve is on the 2nd day, so is the first peak on Bataillon's curve, the great trough on Bataillon's curve presumably corresponds with the low values obtained by Hyman on the 3rd day, and all the later values show a suggestive though not close correspondence. However, from the description given in each case, there is some doubt as to whether the underlying processes are going on synchronously, and doubtless the time of development in the two minnows differs. It may also be significant that Tangl & Farkas's few points for the carbon dioxide output of the trout egg show a slackening of the rise just at the time of Hyman's first trough.

The respiration of the plaice egg (*Pleuronectes platessa*) has been investigated by Dakin & Dakin and by Burfield. Dakin & Dakin made careful analysis of the eggs at two points in their development, at a point between the 8- and 16-cell stages and at 14 days after-

wards. During this period 2000 eggs consumed between 82·086 and 98·00 mgm. of oxygen, and when this amount was compared with the amount of solid matter used up during incubation, the results were very close, for the eggs lost 78·3 mgm. of protein and the oxygen used accounted for between 65·67 and 77·8 mgm. Further consideration of the work of the Dakins will, however, be deferred to the sections on General Metabolism, Energy Sources, etc. Burfield investigated the respiration of plaice eggs with a view to finding out whether the gaseous exchange fell off during a single experiment in the closed chamber, and, if so, why. It is extremely tantalising that he apparently made no record of the age or state of development of his eggs, classing them simply as "young"; his figures which might, therefore, have been useful, as showing differences in respiratory exchange with age, have no value for our main purpose. The fall in the rate of oxygen consumption of aquatic organisms might be due, he argued, to a combination of four factors: (*a*) handling of the animal at the beginning, (*b*) absorption of the available oxygen, (*c*) accumulation of carbon dioxide or other excreta and (*d*) feeding having gone on immediately before the experiment. Factors (*a*) and (*d*) do not operate in the case of eggs, and factor (*b*) was avoided by using a sufficient volume of sea water. Accordingly Burfield found that the third possibility was very important, and was able to depress considerably the rate of oxygen consumption of the developing plaice eggs by adding small amounts of carbon dioxide to the water. They were far more sensitive to this than to reduced partial pressures of oxygen. Urea had no effect. If the eggs were frequently moved so as to prevent accumulation of carbon dioxide in their immediate vicinity no fall in oxygen consumption was detectable, and the amount absorbed during the 1st hour would be a fair average of the values for the succeeding hours. Whitley had already noted that the amount of variation from the normal *p*H which plaice eggs will tolerate is very small indeed, and that a disturbance of the equilibrium towards the acid side is much more fatal than a disturbance towards the alkaline side. These facts fit in together well, but Whitley's work was not altogether confirmed by Hopkins, and the latter actually found that eggs of the trout and perch would not develop properly in the absence of very small amounts of free carbon dioxide. Burfield also occupied himself with the respiratory quotient of the eggs, measuring the carbon dioxide

produced by an indicator method, and the oxygen taken in by the Winkler method. In one case the respiratory quotient was 0·78 and in another case 0·72. We are not informed what the age of the embryos was in these experiments, but the second figure was obtained from a batch which was "younger" than the batch which gave the first value. These respiratory quotients are in fair agreement with the fact now definitely known, that there is a very large expenditure of protein in the egg of the plaice to furnish energy during development.

Kawajiri has studied the embryonic respiration of the Japanese landlocked salmon, *Oncorhyncus masou*. Apparently the oxygen consumption per fish per hour rises steadily until hatching, after which there is a rapid increase, followed by a continued slow rise at much the same slope as before hatching, until the yolk has disappeared, after which time other factors come into play.

The best work on the respiration of the fish embryo is that of Gray, who in 1926 measured the respiration of the brown trout, *Salmo fario*. His experiments did not begin till the 46th day from fertilisation, about which time the fish escapes from the egg-envelopes and swims freely, existing on the stores in its yolk-sac. The graph given in Fig. 126 shows the figures obtained by Gray for metabolic rate. Assuming that the substances combusted were partly fat and partly protein, Gray calculated that the amount of oxygen consumed over the period in question was exactly equivalent to the amount of dry material disappearing from the system. Hayes subsequently studied the respiration of the eggs of *Salmo salar*. It fell from 0·075 gm. oxygen per 1000 eggs per hour on the 40th day to 0·045 on the 65th day.

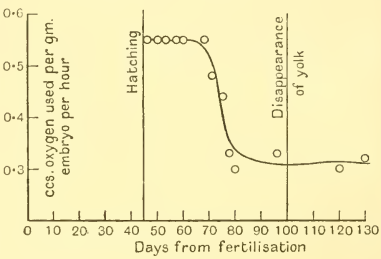

Fig. 126.

In 1928, Boyd, working with the egg of the minnow, *Fundulus heteroclitus*, and using three methods on the same material (Winkler titration, Fenn's modification of Barcroft's manometer, and the Haldane gas analysis apparatus), found that there was a marked rise in oxygen consumption in the egg after fertilisation. This was an

important advance, for although it had always been considered that the Warburg-Meyerhof-Shearer experiments on echinoderm eggs had the stamp of universality about them, there was no evidence that the rise in oxygen consumption at fertilisation held for any other phylum. Plotting the oxygen consumed by her minnow eggs per unit weight in each 10-minute period, against the time, she found that it rose to a peak 60 minutes after fertilisation, at which time it was 17 times the unfertilised egg value. Then, falling past the time of first cleavage (120 minutes) it reached the unfertilised egg value at 210 minutes. Boyd did not take any readings after the 310th

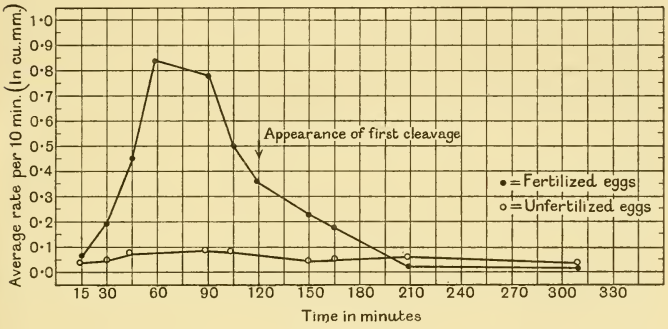


Fig. 127.

minute but a few extra experiments showed her that Scott & Kellicott's rise at the 9th day (beginning of the circulation) could be confirmed. Her curve for the first few hours after fertilisation is given in Fig. 127.

## 4·7. Respiration of Amphibian Embryos

Bataillon was also the first to make quantitative experiments on the respiratory exchange of developing amphibian eggs. His papers already quoted are interesting, in that they form one of the most detailed attempts at correlation between morphological and biochemical data which have ever been made. In this they suffer from two disadvantages, firstly that Bataillon's methods would not be regarded now as accurate although Hyman's work is to some extent confirmatory, and secondly that he made one or two doubtful theoretical

assumptions, the nature of which will presently become clear. In order to understand his point of view, attention should again be directed to Fig. 124, in which his data are presented for the carbon dioxide given off by the embryos of the minnow and the frog during their development. Both show the preliminary rise on segmentation, but the frog curve descends sooner, and then rises steadily to reach a peak about 60 hours after fertilisation, at the same time as the minnow. From that point onwards, the amphibian and the teleost run closely together. The development of the frog, as Bataillon pointed out, is, in fact, more rapid in the early stages. The occlusion of the yolk-plug occurs earlier relatively in the frog than in the minnow, yet the subsequent work of embryonic organisation takes a longer time in the former than in the latter. In the frog, between the initial segmentations and the period of spread of the ectoderm over the yolk there is, as can be seen, a short trough, after which the respiratory activity mounts steadily till the time comes for the closure of the yolk-plug. In the teleost, on the contrary, the period of extension of the blastoderm is short, and is preceded by a well-marked stasis, as if the development was meeting a persistent and not easily conquered obstacle. This once overcome, the extension of the blastoderm over the yolk goes on rapidly, accompanied by the rapid upstroke of the respiration curve. The embryo of the minnow having arrived at the 18th hour is constantly enriching itself with new cells, especially on its under surface. The periblastic elements are exercising a kind of sorting action on the yolk, by which it furnishes an abundance of chromatin material for future cell-divisions. But towards the 30th hour the spider-like forms of cells characteristic of the earlier stage have disappeared, and there is a very sharp boundary-line between the yolk and the periblast, all the cells of which appear to be at rest, very few mitotic figures being visible. In the embryo itself exactly the same state of affairs has come about; the cell-divisions are very rare even on the under surface, where they are mostly to be found at the edges. There is, in fact, a period of temporary cessation of mitotic activity, and of slowing down of developmental rate. Bataillon thought that the reason for this was the heaping up of the layers of cells, as many as fifteen being demonstrable in certain regions, so that the inner ones were cut off from the air, and the outer ones from the nutritive materials of the yolk. In such circumstances the metazoal embryo might be considered to have gone as far as it

could go in the absence of a circulation. Bataillon pointed out that this temporary cessation of mitotic activity corresponded exactly with the trough in carbon dioxide production shown for the teleost in Fig. 124, between the 40th and 50th hours. The mitotic divisions having localised themselves round the edge of the embryonic area, the central part becomes dislocated, or rather raised up, and the deep cells, many of which show amoeboid processes, migrate to the periphery. Between the raised up germinal area and the periblastic layer a fluid appears, thus making a very irregular segmentation cavity, in which the deep-lying cells, finding better conditions, begin to proliferate and form the primitive endoderm. These cellular displacements and migrations, these mechanical difficulties, due to the beginning of extension of surface, said Bataillon, are probably the obstacles which give to that particular stage in teleostean development its peculiar characteristics, among the most striking of which is the trough in the carbon dioxide production. Then, after this stage, the extension of the blastoderm begun by the proliferation of the cells at the edges of the germinal area goes on continuously until the yolk is completely covered. In the case of the amphibian embryo, none of these difficulties arise, for the extension of the ectodermal elements at the animal pole over the yolk-laden cells of the vegetal pole is a relatively simple process.

In commenting on Bataillon's investigations, the resemblance between his curve and that of Hyman must be carefully considered, for, although the time correspondence is sufficiently close to warrant the belief in a real agreement, the descriptions given by the two writers are slightly at variance. *Phonixus*, according to Bataillon, accomplishes its surrounding of the yolk by the blastoderm *after* the great trough, but *Fundulus*, according to Hyman, does it partly before. Again, the curve of Hyman, though of the same shape as Bataillon's, is more lengthened out along the time axis, so that the peaks and troughs do not exactly correspond. Balfour, moreover, does not describe the static condition on which Bataillon lays such stress. It is therefore difficult to appraise Bataillon's results. As regards the theoretical side of his work, it might perhaps be observed that his simple direct correlation between amount of carbon dioxide put out per hour and number of mitoses going on is not altogether satisfactory. We have no evidence that there is a general connection between these two phenomena. Then one might ask why cell-migration should not be

just as much associated with high respiratory rate as mitotic activity. The question is in a confused and unsatisfactory state, and further researches with more accurate methods are greatly to be desired.

Another pioneer worker on the respiration of the amphibian embryo was Godlevski, who published his work in 1900 in connection with the susceptibility of frog's eggs to oxygen want. His technique was rather better than Bataillon's. The data he obtained are shown in Fig. 128, and consist of two smoothly ascending lines composed of rather scattered points.

Nothing further was done on amphibian embryo respiration till 1915, when Bialascewicz & Bledovski attacked the question, using the Winterstein micro-respirometer, a great advance on the technique of the earlier workers. The eggs of *Rana temporaria* were used. Bialascewicz & Bledovski found that, during the first few hours after laying, unfertilised eggs liberated a "neutral gas" which, as far as could be ascertained, was a mixture of oxygen and nitrogen. This doubtless arose from the difference in environment as regards gases between the ovary in the female body and the water outside. One thousand eggs gave rise in this way to a positive pressure of 12·1 mm. 16 minutes after laying, and 1·15 mm. 47 minutes after laying, but thenceforward the pressure was constantly a slightly negative quantity. In order to avoid technical errors which arose naturally from this fact, Bialascewicz & Bledovski compensated the gaseous exchange of one lot of eggs in one vessel by having an identical quantity of eggs from the same female in the other vessel. Another phenomenon seen in the unfertilised eggs was a notable production of carbon dioxide immediately upon laying. Eggs taken from the lower part of the oviduct and brought straight into the micro-respirometer eliminated large amounts of carbon dioxide, presumably because the tissues of the frog with which the eggs had previously been in gaseous equilibrium were saturated with carbon dioxide. This carbon

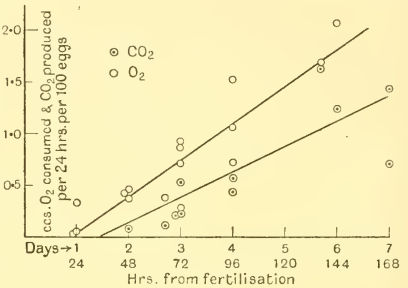


Fig. 128.

dioxide output declined rapidly after the eggs had been taken from the oviduct or had been laid, and by 100 minutes had reached a steady low level. One thousand eggs 50 minutes after laying produced upwards of 20 c.mm. of carbon dioxide per minute, but after the 100th minute was reached, their steady level was about 1 c.mm. carbon dioxide per minute. McClendon's centrifugation experiments already referred to showed that 16 per cent. of the frog's egg was "clear and protoplasmic", 6 per cent. was fatty or oily and 78 per cent. was yolk. As an average egg of *Rana temporaria* weighs 3·43 mgm. (Bialascewicz & Bledovski), the amount of protoplasm in it would weigh 0·55 mgm., or in 1000 eggs 550 mgm., and this amount at 1 c.mm. carbon dioxide per minute (wet weight) would be 0·182 c.mm. carbon dioxide per 100 mgm. protoplasm which compares interestingly with Rapkine's 0·225 c.mm. carbon dioxide per 100 mgm. in the sea-urchin's egg. But this calculation is not very significant, owing to the manifold uncertainties involved in taking data from many authors on different material in different conditions. It does perhaps show a similarity between the metabolic rate of the unfertilised egg of echinoderm and amphibian. The conclusion from this part of Bialascewicz & Bledovski's work was that during life in compact masses in the oviduct the eggs accumulate large quantities of carbon dioxide, which has to be eliminated when the eggs are brought into an atmosphere containing a much-diminished concentration of carbon dioxide. If this process were to go too far fertilisation would become impossible, as Bialascewicz & Bledovski showed by subjecting the unfertilised eggs to atmospheres of pure carbon dioxide for as little as 2 hours, and these findings do indeed go a long way towards explaining the phenomenon of prematuration

The average results for oxygen uptake and carbon dioxide production of unfertilised and fertilised eggs worked out as follows:

| | Carbon dioxide liberated by 100 eggs per hour (c.mm.) | Oxygen used up by 100 eggs per hour (c.mm.) | Respiratory quotient |
|---|---|---|---|
| Unfertilised | 5·77 | 9·03 | 0·639 |
| ,, | 4·18 | 7·69 | 0·543 |
| ,, | 2·90 | 4·88 | 0·590 |
| Average ... | 4·28 | 7·20 | 0·591 |
| Fertilised | 6·28 | 10·00 | 0·628 |
| ,, | 6·57 | 12·86 | 0·511 |
| ,, | 6·07 | 8·90 | 0·682 |
| Average ... | 6·31 | 10·58 | 0·607 |
| [Gastrulation | 24·4 | (single experiment of Saunders)] | |

It is very interesting to note that the respiration shows a distinct increase after fertilisation, the extreme instance being a rise of two and a half times in the case of carbon dioxide and of nearly three times in the case of oxygen.

Turning now to the respiratory quotients, it is evident that there is not much difference before and after fertilisation, a fact which is interesting in view of the constancy of the calorific quotient in echinoderm eggs. Bialascewicz & Bledovski admitted the difference between 0·60 and 0·72 the theoretical fat respiratory quotient, but claimed quite justifiably that in many other cases where vigorous catabolism of fatty acids is known to be going on, the respiratory quotient is often below 0·7. At the same time, they saw in the lowness of it in this case evidence for a simultaneous activity of hydrolytic processes, both before and after fertilisation, though they did not give any more detailed indication of the part they supposed these to be playing in the metabolism of the embryo.

Bialascewicz & Bledovski went on to measure various entities in the egg and the early stages of development. Weighing, they found, presented great difficulties, so they measured the diameter microscopically, calculated its surface and its volume which, when multiplied by the specific gravity (1·102), gave the mass. From these investigations the following figures resulted:

| | Oxygen taken up by unfertilised eggs | | |
|---|---|---|---|
| | Per 1000 eggs (c.mm.) | Per 1 sq. metre surface (c.c.) | Per 1000 gm. egg-weight (c.c.) |
| Averages of 8 experiments ... ... ... | 74·5 | 7·1 | 21·6 |
| Percentage scattering of individual observations | 33·0 | 19·2 | 18·0 |

From these measurements it appeared that the smallest deviation from the average value resulted when the oxygen taken up was referred to the actual weight of the eggs, though evidently the surface is nearly as good a measure. This shows that the oxygen uptake depends not so much on the quantity of individuals measured as on their surface, or even more, on their weight. The cubic centimetres of oxygen absorbed per kilo of unfertilised egg-weight may be compared in an interesting way with the cubic centimetres of oxygen absorbed per kilo of adult frog (Bohr); the figures are respectively 21·6 and 261·8, so that the metabolic rate is evidently far higher in the adult frog than in the unfertilised frog's egg.

Bialascewicz & Bledovski next studied the intake of oxygen during the early stages of development, and their results are shown in Fig. 129. The curve rises smoothly during segmentation and gastrulation, etc., but about the 150th hour attains a plateau. This plateau is maintained as long as the tadpoles are kept without food, but when they begin to eat the respiration rises in amount again. Bialascewicz & Bledovski rightly laid special stress on their confirmation of Warburg's results, in that the respiration does not rise *pari passu* with the increase in general mass of nuclear substance. In absolute amounts the values for oxygen intake of Bialascewicz & Bledovski are almost double those of Godlevski, but all these investigators agree in the general upward trend of the curve and the absence from it of the undulations found by Bataillon. They found that the rising part of the curve, i.e. the plateau, could be expressed by an equation for a parabola, before i.e. $x = kt^2 + a$, where $x$ is the amount of oxygen taken up by a given number of eggs during time $t$, $a$ the value for unfertilised eggs, and $k$ a constant. This rise then proceeds in a manner directly proportional to the square of the developmental time. Bialascewicz & Bledovski applied the same equation to the figures obtained by Meyerhof for the respiration of the sea-urchin and by Bohr & Hasselbalch for the chick. In the case of the frog, the constant was 0·0139, and the agreement between observed and calculated values was very fair, as follows:

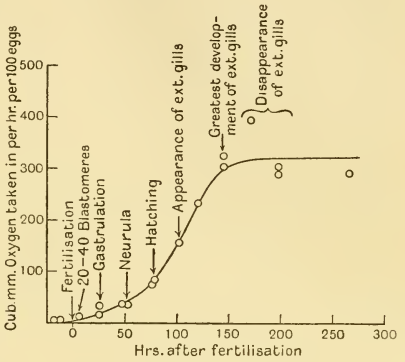

Fig. 129.

| Hours after fertilisation | Oxygen used up by 100 eggs per hour | |
|---|---|---|
| | Observed | Calculated |
| 0·0 | 5·2 | 5·2 |
| 25·0 | 16·4 | 13·9 |
| 52·0 | 33·1 | 40·8 |
| 78·5 | 77·3 | 66·8 |
| 120·0 | 231·3 | 191·5 |
| 149·7 | 324·3 | 316·7 |
| 170·0 | 392·2 | 406·9 |

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The matter was then taken up by Parnas & Krasinska, who published their results in 1921. On many points their work led them to other conclusions than those of Bialascewicz & Bledovski, for they could not find respiratory quotients corresponding to the combustion of fat, and they did not obtain perfectly regular curves for oxygen consumption. They used as their principal method the Barcroft manometer technique, and their experiments seem to have been better carried out than those of any of their predecessors. They worked on the eggs of three species of amphibia, *Rana temporaria*, *Bufo variabilis*, and *Rana esculenta*. The experiments included more elaborate controls than had before been made, e.g. it was ascertained that the presence or absence of the gelatinous coverings made no difference to the respiration of the embryos in any stage, and that the development was normal in all the experiments, irrespective of the partial pressure of oxygen within wide limits. After 70 hours at 15°, the embryos which had been in a pure oxygen atmosphere were slightly more advanced than those which had been in the air, but at 11° this difference was hardly noticeable, although the diffusion of the gases would hardly be affected at all by such a change of temperature. Experiments were carried through at the higher temperature when it was desired to follow a long period, but at the lower temperature when the details of the early stages were under examination.

Parnas & Krasinska found that the oxygen consumption of *Bufo vulgaris* eggs at 14° in air before fertilisation was 0·09 c.mm. per egg per hour, but after fertilisation 0·34 c.mm. per egg per hour, a rise of about four times, rather more than had been found by Bialascewicz & Bledovski on the frog. In pure oxygen the rise occurred just the same, and to the same extent, though the absolute figures were higher. Parnas & Krasinska regarded the unfertilised egg as a dying cell, in view of the fact that, at a definite time after laying, it loses its capacity for being fertilised, and they suggested that the asphyxial conditions which had been shown by the earlier workers to hold in the oviduct were important as conserving the eggs in a state of suspension, it being impossible for any great amount of metabolism to go on in them before laying.

Typical graphs of Parnas & Krasinska's results are given in Figs. 130, 131 and 132. The first of these shows the oxygen uptake of the eggs of *Rana temporaria* for the first 100 hours after fertilisation;

this curve is not an increment curve, but shows the total amounts consumed up to each time point, in cubic millimetres of oxygen.

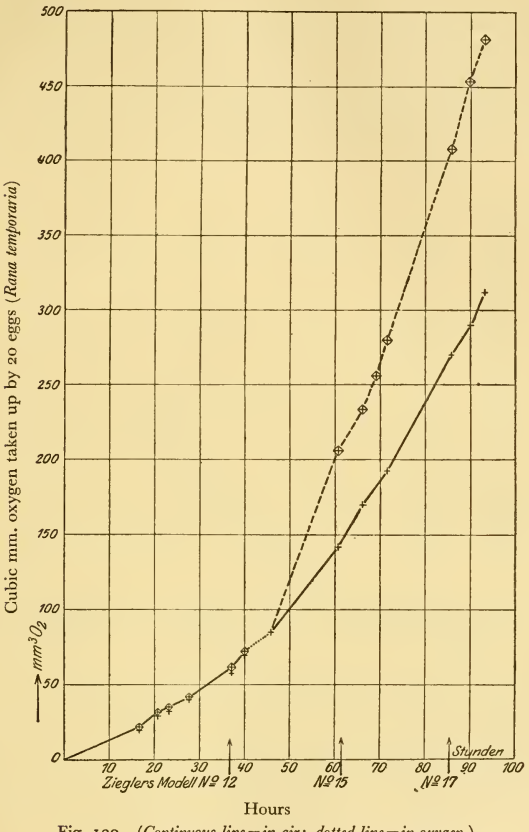



Fig. 130. (*Continuous line = in air; dotted line = in oxygen.*)

The second figure shows the same thing for the eggs of *Rana temporaria* for the first 70 hours after fertilisation, and in the third figure is shown an increment curve, i.e. the amounts of oxygen consumed per hour by 1000 eggs. As is evident, the rise in respiratory rate

is not regular, but has certain definite points of transition, and tends to approximate rather to a series of straight lines than to a segment of a curve. The findings of Parnas & Krasinska, then, were in a sense a return to the original interrupted curves of Bataillon. The respiration of the amphibian embryo, said Parnas & Krasinska, during the segmentation, morula, and blastula stages, is proportional to the time passed and uniformly rising. The increase in the number of cells is certainly accompanied by an increase in the respiration. But at

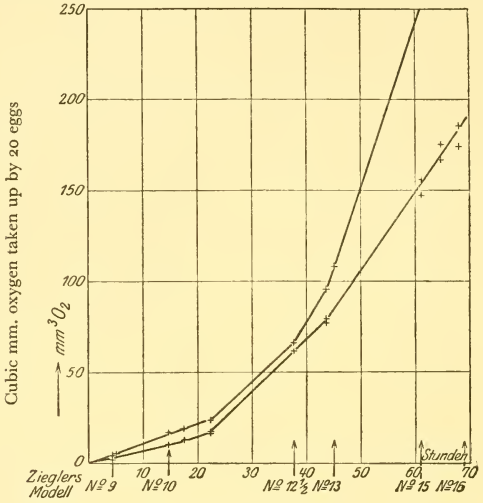



Fig. 131. (*Upper line = in air; lower line = in oxygen.*)

the time of gastrulation, there is a marked rise in the gaseous exchange (see Fig. 132), and a further powerful acceleration of it is seen at the time of the formation of the medullary plate, i.e. after the embryo has formed its longitudinal axis. "The first cleavage processes", said Parnas & Krasinska, "and the dividing of the embryo into potentially different cells proceeds with a uniform amount of respiration, but the differentiation of the germ-layers first of all, and then, the formation of structurally and chemically different cells, are accompanied by enhanced metabolic intensity and therefore by an increased respiration. After the neurula has been formed there is little

or no change in the respiration until the external gills appear, at which stage there is a third increase of oxygen-uptake. The succeeding period is again marked by a uniformity of rise." The three critical points in amphibian development, then, according to Parnas & Krasinska, are (i) gastrulation, (ii) formation of medullary plate, neural groove, etc., and (iii) appearance of external gills. These relations can best be seen on the increment curve in Fig. 132, where the breaks at gastrulation and the formation of the neural groove can be seen well.

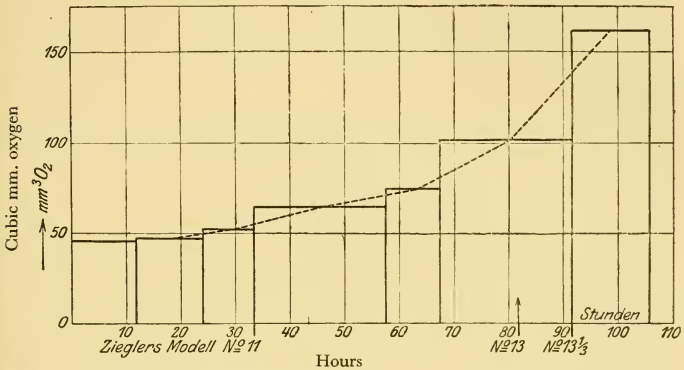



Fig. 132.

Parnas & Krasinska considered the question of whether their sudden increases in respiration could be accounted for by changes in the surface-volume relation or other causes of non-metabolic origin. At the neurula stage, for instance, when the spherical form of the embryo is abandoned, a greater oxygen consumption might be supposed to be due to the consequent increase of surface. But they concluded that the operation of such factors did not account for their results, since gastrulae respired no more in pure oxygen than in air.

Parnas & Krasinska suggested as the cause for the low respiratory quotients found by Bialascewicz & Bledovski processes in which oxygen was combined in the materials of the cells under construction —the same explanation as Meyerhof had already advanced. If they were indeed accurately measuring the carbon dioxide output of the

embryos, and such factors as alkali reserve, etc., were not exerting too great an effect, then the Meyerhof theory is certainly more satisfactory than the one suggested by Bialascewicz & Bledovski. But it remains to be shown that absolutely all the carbon dioxide produced was being successfully measured. That much fat is burnt during amphibian development was definitely denied by Parnas & Krasinska on the basis of their actual estimations of fat and protein during the embryonic period, but this aspect of their work must be reserved for discussion later. According to their view, the segmentation period involves no more than a distribution among cells of protoplasmic constituents already in existence in the egg, and it is not until the separation of the embryo into the three germ-layers that intensive chemical work begins to take place. It is this that leads to rise in respiration.

Frog's eggs, it seems, can be anaesthetised, and Irwin has studied the consequent variations in their carbon dioxide output.

## 4·8. Heat-production of Amphibian Embryos

Only one examination of the heat-production of amphibian eggs exists in the literature, namely, that of Gayda, who described a differential microcalorimeter of great accuracy which could be used for small objects. With this instrument he measured the heat-production of the eggs of the toad, *Bufo vulgaris*, throughout their development from fertilisation to hatching. Ruffini had previously ascertained that small amounts of water in which the eggs of *Bufo vulgaris* were developing had usually a temperature of from 0·5° to 0·6° at 10° and from 1·0° to 1·5° at 20° higher than control quantities. Gayda confirmed many other workers on other material by finding that the heat-production did not rise *pari passu* with the increase in nuclear material or in number of blastomeres. Fig. 133, taken from Gayda's paper, shows the curve which he constructed from all his average results, where gram calories of heat evolved per gram of embryo and larva per hour is plotted against time, i.e. days from fertilisation. The arrow pointing downwards marks the point of hatching, and towards the 120th day metamorphosis begins. The shape of the curve is very regular and striking. Rising smoothly from the moment of fertilisation, and so continuing unaffected by the hatching process, it reaches a blunt peak at about the

20th day, after which it slowly declines, never falling, however, below about a third of its maximum value. This curve is, of course, a true measure of metabolic rate, and obviously fits in well with the work of Parnas & Krasinska, and of Bialascewicz & Bledovski. We here see for the first time a possible reconciliation between the apparently conflicting behaviour of the metabolic rate in various types of embryo. As far as can be ascertained at present, the metabolic rate in the echinoderm and the amphibian embryo rises during its ontogenesis, yet there is ample evidence, as we shall see later, that the metabolic rate of the avian embryo consistently falls, at any rate from the 4th or 5th day of incubation onwards.

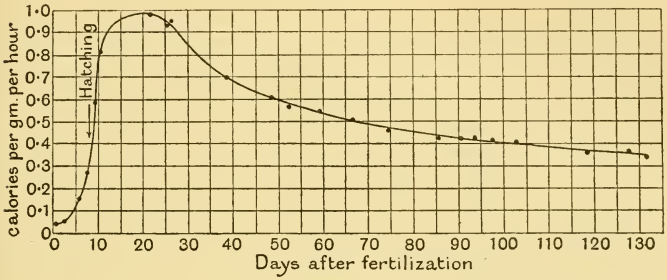



Fig. 133.

Possibly the curve for the heat-production of the toad embryo gives the clue in suggesting that in all embryos there is a point at which the metabolic rate is higher than at any other time. The investigations of the extremely early stages in the echinoderm egg which we have been discussing have on this view revealed only the upward stretches of this curve, while the work on the chick embryo, which it must be remembered has completed its gastrulation before the egg is laid at all, has shown us the descending part of the curve. In the mammal, moreover, the curve for heat-production per gram per hour follows a peaked course; for instance, the work of Wood and his collaborators has revealed very accurately the time at which this takes place in the pig. Two points must not be lost sight of in this discussion, firstly, that, for questions such as these, no hard-and-fast line can be drawn between embryonic or foetal growth and post-natal growth; the act of birth or hatching may be relatively

unimportant, and the fundamental waves of metabolism, growth, and differentiation, may pass through the individual organism without paying much attention to it. Secondly, although it is our duty to regard as many kinds of embryo as possible as special cases only of a few profound and general rules, we cannot escape the fact that their origin is very different, and it may not at present be possible to speak of holoblastic and meroblastic eggs, for instance, on the same basis. Thus the anomalous case of *Ascaris* eggs, in which there is no rise of metabolic rate during segmentation, etc., must be remembered. I shall return to these questions at the end of this section, when the data for the bird and the mammal have been presented.

Gayda's curve shows that shortly after fertilisation 1 gm. of toad embryo liberates 0·037 gm. cal. per hour, while at hatching it liberates about 0·30 gm. cal. per hour, and at its maximum (20th day after fertilisation) it liberates as much as 0·97 gm. cal. per hour. Gayda did not himself calculate any calorific quotients, for he did not himself make any estimations of oxygen uptake, nor had at that time Parnas & Krasinska's work on amphibian embryos been published. Unfortunately, although they worked with *Bufo vulgaris*, none of their published protocols refer to that organism, but all to *Rana temporaria* and *Rana esculenta*. It is therefore not possible to put the figures together. The total amount of heat given out by 1 gm. of embryo (wet weight) throughout the embryonic period (fertilisation to hatching) was 30·276 gm. cal., and the corresponding quantity for 1 embryo was 0·1207. These values are the "Entwicklungsarbeit" of Tangl, relative and simple respectively (see p. 950). Gayda pointed out that the figure of about 30 gm. cal. was not nearly so great as the corresponding values for the chick found by Tangl & von Mituch, and for the silkworm by Tangl & Farkas, of about 900 gm. cal., but, on the other hand, it was about equal to Fauré-Fremiet's figure for *Sabellaria alveolata*. These questions of energetics will be dealt with fully in the section on that subject. Between hatching and the end of metamorphosis 1668 gm. cal. were given out per gram wet weight, and 67 gm. cal. per larva. This covered 123 days. Thus the average heat output per gram wet weight per day before hatching was 3·75 gm. cal., and the average afterwards was 13·58, a striking result of the peak which occurs after hatching.

Gayda, attempting to explain the slow fall in heat-production and metabolic rate after the 20th day, discussed the relations between surface and volume. As the larva grows in volume so its surface must proportionately diminish. What is so striking about the peaked curve for metabolic rate is that in the very earliest stages of development, while segmentation and gastrulation are proceeding, the heat-production per gram per hour is increasing in spite of the fact that every moment the surface is diminishing in proportion to the volume and the weight. After the main inflection in the curve a simple surface heat-radiated relation is conceivable, but not before it. It is probable, of course, that the dermal surface is not the active surface, or rather not completely coterminous with it. An immense field of study exists in the determination of the surfaces in the growing embryo and the identification of the active one. Gayda found that, after the 20th day, if the gram calories produced were related to the surface of the embryo (calculated by the $\sqrt[3]{W^2}$ formula) the result was almost a constant, though at first there was some divergence. Thus about the 25th day 100 sq. mm. radiated 0·211 gm. cal. per hour, but on the 97th day 0·171 gm. cal., and on the 131st day 0·169 gm. cal. In fact, the gram calories liberated per square millimetre per hour form a curve which declines from the 25th day; this is represented in Fig. 134. If it is compared with Fig. 166 *a*, in which the calories radiated from 1 sq. metre per hour are plotted against the age in the case of the pig and the human being, the resemblance is very striking. I shall return to this point. Gayda himself did not see anything important in this peak, however, and considered that it was probably due, on the one hand, to the change in shape of the embryo from spherical to axial, and, on the other hand, to the first swimming movements of the embryo about the time of hatching, believing that, if a constant could be introduced into the calculations to allow for such changes, the peak would entirely disappear. This may or may not be the case, and it is

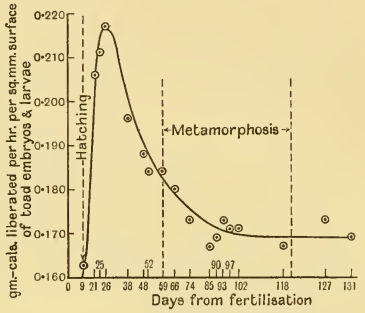



Fig. 134.

also necessary to remember that the unabsorbed yolk-mass will in the early stages be included in the weight estimations, though it cannot be counted as thermogenetic tissue. It is easy to understand the decline in metabolic rate with advancing age, for the surface, i.e. the means of exit from and entrance to the body, does not grow as fast as the weight, but it is difficult to understand, on the view held by some physiologists that thermolysis is the cause of thermogenesis, how the embryo can have an increasing metabolic rate in the early stages—as it assuredly does—when every moment the surface/volume ratio is diminishing. The factors controlling the production of heat must be sought somewhere within the body rather than at the surface.

Gayda also discussed the interesting relations that exist between the heat-production and the time required to double the weight. Curves for these values are shown in Fig. 135. The S-shaped nature of the curve is striking, but perhaps the lowering at the older stages is brought about by metamorphosis, and so lies out of the strict part of this discussion. During the greater part of the larval period, both before and after feeding has commenced, however, the parallelism between the two curves is close. Obviously, the less heat that is evolved during the doubling of the weight, the more efficient will be the embryo or larva, and the more economically the turnover will be progressing. Fig. 135 shows that the least heat is evolved in the earliest stages, i.e. shortly after hatching, so it must be concluded that the greatest efficiency exists then. The most inefficient point would appear to be at the weight of 40 mgm. Whether it is significant that just at this point feeding begins is not clear. These relations are the direct opposite of what has been found to hold in the case of the chick, which at the 3rd day of incubation appears to be very inefficient, but which attains a maximum efficiency a few days before hatching. A full treatment of this point is given in Section 7·5.

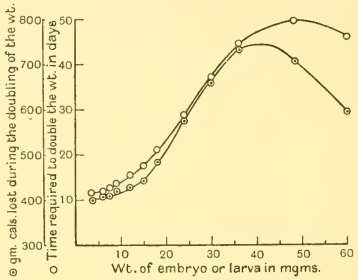



Fig. 135.

Gayda also calculated the temperature coefficient of the heat production, and found that it worked out at an average of about 2·10,

but his data at different temperatures are hardly sufficient to allow of the calculation of a temperature characteristic. The total quantity of heat lost, however, remained quite constant during periods of doubling of weight at all temperatures at which normal development proceeded; a finding which was later to be confirmed very fully on the frog by Barthélemy & Bonnet using bomb calorimetric methods, as will be related in the section on energy changes in embryos. Gayda had expected that he would find the total amount of heat given out to be the same at all temperatures, for, as Chambers and Terni had shown, the amount of growth in frog larvae was the same, only metamorphosis supervened earlier at the higher temperatures than at the lower ones. Finally, Gayda compared the heat production of the eggs with adult frogs. The latter evolve 0·45 gm. cal. per hour per gram on an average, as against the maximum of 0·98 gm. cal. per hour per gram at the 20th day from fertilisation.

## 4·9. Respiration of Insect Embryos

The first organism of this kind which was examined was the silkworm, *Bombyx mori*. Apart from early work by Duclaux, the first papers were those of Luciani and Luciani & Piutti, who estimated quantitatively the gaseous exchange of the eggs. The silkworm embryo has a complicated course to pursue, for the egg is laid in the late summer or autumn, and the first week after fertilisation is passed before the hibernation period can be said to have begun. During this time the colour changes from pale yellow to greyish brown. Throughout the winter the egg remains in a sort of latent state, but when the spring begins the developmental process is suddenly released, and 11–14 days are sufficient for hatching. A similar quiescent period or "diapause" is observable in grasshoppers and may be shortened or lengthened, according to Bodine, by raising or lowering the temperature. At high temperatures the quiescent period may only be represented by a trough but it cannot be abolished altogether[1].

Luciani & Piutti were not able to confirm the preliminary results of Luciani himself, that during the hibernatory period the eggs could live without oxygen, but found instead that the respiration at that time was directly proportional to the partial pressure of oxygen.

[1] Conditions in orthopteran development are rather complicated, and for the details reference should be made to the memoirs of Bodine. Respiratory Quotients of 0·7 to 0·8 seem to be usual in this material.

Excessive amounts of oxygen exerted a marked toxic action. In normal development (after the winter diapause was quite passed through), the amount of carbon dioxide given out per day per kilo of eggs rose quite regularly until at hatching the value was 259 times what it had been initially. In Fig. 136, constructed from the data of Luciani & Piutti, the graph of this process is given; it does not, of course, represent metabolic rate, for nothing is known of the weight of protoplasm present at different stages. During the whole period 55·118 gm. of carbon dioxide were given off by 1 kilo of eggs, representing a loss of 1·5 per cent. of the carbon originally present. Throughout the whole period, including the time of hibernation, the behaviour of the eggs as regards weight was variable, for sometimes they became heavier, owing to absorption of water in a humid atmosphere, and sometimes they lost weight, owing to exhalation of water in a dry atmosphere. Physiologically they were apparently unaffected, except that more carbon dioxide was evolved weight for weight during the wet periods than during the dry ones[1].

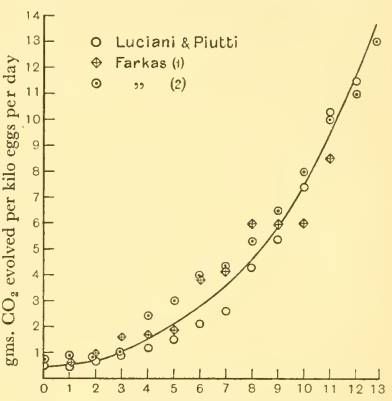


Fig. 136.

The respiratory quotient gave a curious result, for while it was about 0·97 at the beginning of the developmental period it then rose steadily, passing unity when between a third and a quarter of development had been completed, and rising to 1·305 by the time of hatching. Luciani & Piutti considered that carbohydrates were being combusted throughout development.

Some of the less important conclusions of Luciani & Piutti were

[1] Ashbel, in later work, obtained curves very similar to Bodine's, showing first a somewhat intense respiration, which dies away after 4–5 days, giving place to the quiescent period's almost imperceptible gas-exchange. The silkworm egg respires much less before than after fertilisation (see p. 640) and gives out a gas, probably $CO_2$, for some time after laying, even when not fertilised (see pp. 712 and 819).

confirmed by Quajat in 1899, who, however, made no measurements of the respiratory quotient, and in 1903 Farkas went into the matter again. He used the same technique as that of Bohr & Hasselbalch on the chick, and his experiments did not begin until about a fortnight before hatching, at which time the sudden rise in the respiration occurs. Farkas' figures were in good agreement with those of Luciani & Piutti; thus in his experiments 1 kilo of eggs just before hatching evolved 8·7 gm. of carbon dioxide per day,

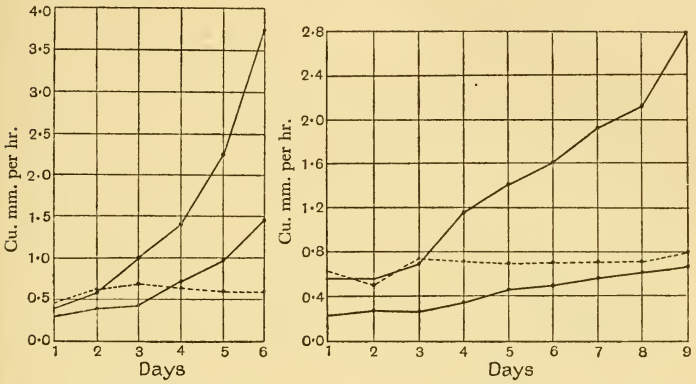



Fig. 137. (*The upper solid line in each figure represents the $O_2$ intake, the lower solid line the $CO_2$ output, and the dotted line the R.Q.*)

while in theirs 1 kilo of eggs at the same time evolved about 10 gm. A curve constructed from his data is given in Fig. 136. Unfortunately, he did not make any determinations of the oxygen uptake, so no respiratory quotient was calculated.

In 1925 Fink made a careful examination of the respiratory exchange of 10 different kinds of insects, mostly beetles, using the Krogh differential manometer and Jacobs' modification of the Haas colorimetric method for studying carbon dioxide elimination. Figs. 137 and 138, taken from Fink's paper, all show the curves obtained for carbon dioxide output during the embryonic development of beetles. In each case there is what Fink calls a preliminary "formative period", followed by a continuous rise in respiration (grams of

carbon dioxide excreted per gram egg per hour—therefore not metabolic rate). In *Crioceris asparagi*, *Hylemyia chortophila*, and *Leptinotarsa decemlineata* (the asparagus beetle, the seed-corn maggot, and the potato beetle respectively) the formative period is evidently very brief, not occupying more than a single day, but in *Popillia japonica* (the Japanese beetle) it lasts for more than 6 days, and, when the respiration does rise, it rises very steeply. The formative period mentioned by Fink perhaps corresponds to the early flat part of the curve in the case of the measurements on the silkworm egg by Luciani and Farkas, but, as in nearly all cases (e.g. the chick)

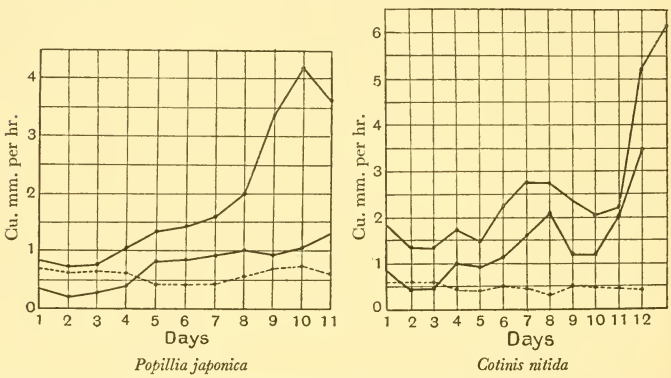



Fig. 138. (*The upper solid line in each figure represents the $O_2$ intake, the lower solid line the $CO_2$ output, and the dotted line the R.Q.*)

respiration rises in a curve convex to the abscissa, it is questionable what definite meaning can be attached to the "formative period". Fink suggested that there was a correlation between short formative period and the deposition of eggs on foliage or soil surface (examples would be *Leptinotarsa* and *Hylemyia*) on the one hand, and between long formative period and deposition of eggs at some depth below the surface of the soil (examples: *Popillia* and *Cotinis nitida* (the green June beetle)).

Fink drew a very interesting comparison between embryonic respiration and the respiration in metamorphosis when he set side by side the carbon dioxide and oxygen turnover per gram per hour during the two processes.

Table 80.

| | Mgm. carbon dioxide output per gram per hour | | Mgm. oxygen intake per gram per hour | |
|---|---|---|---|---|
| | Egg | Pupa | Egg | Pupa |
| *Leptinotarsa decemlineata* ... ... | 4·89 | 3·43 | 4·05 | 1·82 |
| *Hylemyia cilicrura* ... ... ... | 12·30 | 7·89 | — | — |
| *Popillia japonica* ... ... ... | 18·90 | 3·87 | 3·85 | — |
| *Hippodamia convergens* ... ... | — | — | — | 1·57 |
| *Epilachna borealis* ... ... ... | 11·00 | 5·10 | 1·67 | — |
| *Crioceris asparagi* ... ... ... | — | — | 3·46 | 2·72 |
| *Cotinis nitida* ... ... ... | — | — | 3·27 | — |
| *Macrocentrus ancylivora* ... ... | — | — | — | 3·17 |
| *Ancylis comptana* ... ... ... | — | — | — | 5·10 |

It is evident that with one or two exceptions the intensity of respiratory exchange is much greater in the egg than in the pupa, so that this at any rate marks a definite difference between embryonic development and metamorphosis. The respiratory quotients were always found to be low. Thus *Anasa tristis* (the squash bug) gave an initial value of 0·521 rising on the third day to 0·732, and maintaining itself at that level through subsequent development, while the respiratory quotient of *Leptinotarsa* ranged daily from 0·512 to 0·68. *Cotinis nitida* went even lower, seldom rising above 0·524, and even dropping for several days to 0·413. *Popillia japonica* began with a respiratory quotient of about that level, but rose in the last few days of development to 0·732. No explanation is available for these curious quotients; evidently either an abnormally small amount of carbon dioxide was escaping or an abnormally large amount of oxygen was being taken in. There seems no reason, from a technical point of view, to doubt the accuracy of Fink's analyses, so it is probable that in these insects whatever combustion processes are going on are obscured by transformations of another order, such as the passage of fat into carbohydrate. This increase of highly oxygenated material at the expense of less oxygenated material leads, in the well-known case of the hibernating marmot, to very low respiratory quotients, and might perhaps be due in the case of these beetles to the formation of chitin.

Melvin later estimated the oxygen uptake during the embryonic development of a number of insects, using the Krogh micromanometer. The organisms employed were *Anasa tristis*, the squash bug; *Tropoea luna*, the luna moth; *Samia cecropia*, another moth; and *Pyrausta ainsleii*, the smartweed borer. By a method not stated, Melvin was able to measure the weight of the shells of the eggs and

this he deducted from the weight of the whole egg in his calculations —it amounted to an average of 25 per cent., for the exact figures see p. 322. The oxygen consumption expressed in cubic millimetres of oxygen per gram egg-contents per hour rose very steadily throughout the incubation period, and this, of course, is exactly what was found by the workers on the silkworm egg. It gives us no information about the metabolic rate of the embryonic cells, and is simply a reflection of the increase of organised living matter with corresponding decrease of the non-respiring yolk. Melvin expressed disagreement with Fink's hypothesis of the formative period in relation to foliage eggs and earth eggs, and his results certainly do not support it. He also made the interesting observation that temperature had almost no effect on the respiration at the beginning of development though it acted powerfully at the end. Thus a rise of 20° in the external atmosphere gave a rise of 0·01 mgm. oxygen per gram per hour on the 1st day of incubation and a rise of 1·92 mgm. oxygen per gram per hour on the last day.

## 4·10. Respiration of Reptile Embryos

Only one investigation has been made of the respiration of the reptilian egg. Bohr in 1904 worked on the snake, *Coluber natrix*, from this point of view, having been instigated to do so by the results he had already obtained on mammalian and bird embryos, and by the researches of Pembrey, Gordon & Warren on the development of heat regulation in the chick. These snake's eggs were developing under natural conditions in a heap of leaves at a temperature of about 29° and in an atmosphere which, on examination, turned out to have only 4·7 per cent. of oxygen and as much as 13·8 per cent. of carbon dioxide. Bohr was able to incubate them artificially by keeping the air very humid, though he did not attempt to reproduce an atmosphere of that composition. His figures are shown in Fig. 139 graphically. About three

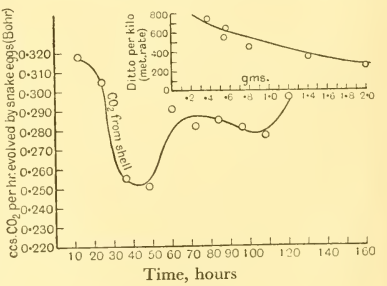



Fig. 139.

times as much carbon dioxide was given out at 28° as at 14°, so that the $Q_{10}$ for respiration in this egg closely approached that for developmental rate (see p. 508). The metabolic rate values worked out as follows:

| Weight of embryos | C.c. carbon dioxide per kilo per hour | |
|---|---|---|
| | At 28° | At 15° |
| 0·38 | 724 | — |
| 0·54 | 548 | 150 |
| 0·56 | 659 | — |
| 0·81 | 467 | — |
| 1·40 | 362 | — |
| After hatching (3·8) | 240 | 90 |

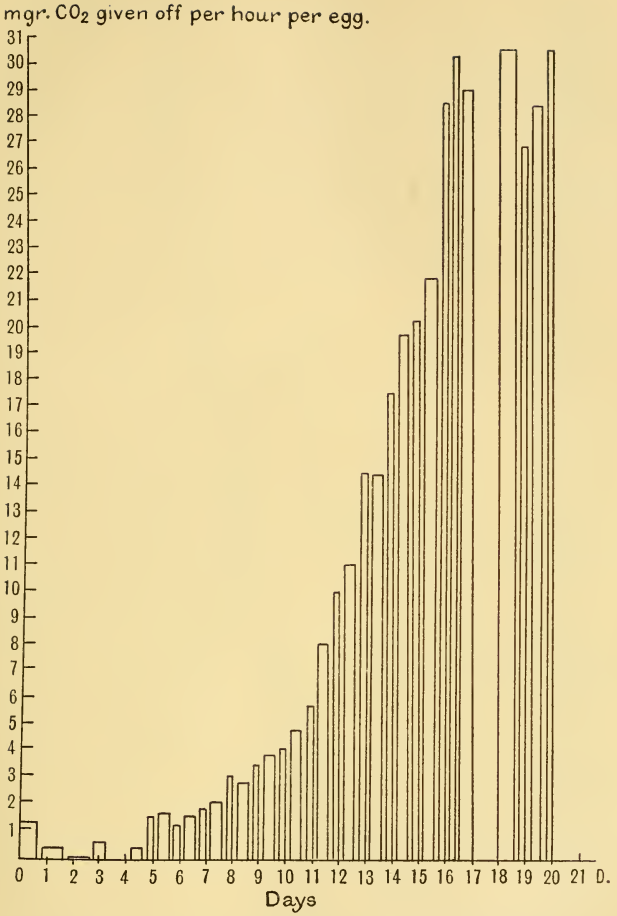
so that there was a very obvious decline in the respiration intensity with increasing age. In view of the fewness of the figures, no stress can be laid on the shape of the curve, but it does not seem to follow the course suggested for metabolic rate curves by Murray, i.e. slow decline at first, followed by greater rapidity of fall. The table shows also that the rule of decline applies to eggs incubated at 15° as well as at 28°. Here we have a case like that of the chick, where the earliest stage which it has so far been possible to examine has the highest metabolic rate of all. If figures could be obtained for the snake or the chick at about the time of gastrulation a great advance would have been made. The respiratory quotient of the snake's egg was shown by Bohr to remain in the close neighbourhood of 0·9 throughout development, so that he concluded there was a dominant complete combustion of carbohydrate material.

## 4·11. Respiration of Avian Embryos in General

I have spoken already about the earlier researches on the respiration of the avian embryo. The modern period in this subject begins in 1900, when Bohr & Hasselbalch published the first of their series of classical papers on the evolution of carbon dioxide, the absorption of oxygen and the heat-production of the hen's egg. Their first paper was concerned exclusively with the production of carbon dioxide during the incubation period, for they wished to study the relation of metabolic rate to age and weight, a correlation the importance of which none of the earlier workers, such as Baumgärtner or Pott & Preyer had appreciated. Their apparatus consisted of a thermostat chamber in which the egg was placed connected with a chain of absorption-tubes, etc. Experiments with empty egg-shells from

freshly laid eggs showed them that an appreciable amount of carbon dioxide could be given off from bicarbonates in the shell—a finding that might have been expected in view of the fact that the oviduct of the hen is probably saturated with carbon dioxide, unlike the air outside. Compare with this result the work of Bialascewicz & Bledovski on amphibian eggs[1]. These experiments with shells alone provided Bohr & Hasselbalch with a correction which they introduced into the values obtained for fertile normally developing eggs. This correction, while negligible in later stages of development, was very important in the early stages where the respiration of the embryo is small. More often they got over the difficulty by keeping fertile eggs at room temperature in a current of carbon-dioxide-free air until they gave off no more of the gas. Controls carried through for 3 weeks on infertile eggs showed that Pott & Preyer had been wrong in their conclusion that infertile eggs gave off notable quantities of carbon dioxide, for, on the contrary, the amount given off was remarkably small, varying from 0 to 5 mgm. per 24 hours. Then they proceeded to the experiments with the developing embryos, obtaining the diagram shown in Fig. 140. The varying width of the columns is a measure of the length of time the experiment was conducted; thus in one or two cases, it was as much as 24 hours, but in the majority only 4 or 5. A glance at the graph shows the initial production of gas from the shell, the steady rise from the 2nd day onwards, and the plateau which Bohr & Hasselbalch always got from the 17th to the 21st day. Their next interest was the variation in the respiration intensity. For this it was necessary to make weighings of the embryos, for at that time the fragmentary values of Falck were all that were available. The numerical results which Bohr & Hasselbalch obtained are shown in Appendix 1, and agree well enough with those got by later observers, but they made the interesting correlation that the curve for total weight (not weight increments) went up in exactly the same manner as the curve for the carbon dioxide excretion in cubic centimetres per 24 hours (i.e. increments of respiration). This is shown in Fig. 141 taken from their paper, and it should be noted that the agreement is rather better after the 9th day than it is before it. The metabolic rate values are shown in Fig. 142 constructed from their data. A sharp descent brings the metabolic rate down to what is

[1] And see pp. 712 and 819.


mgr. $CO_2$ given off per hour per egg.
31
30
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16
15
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Days

Fig. 140.

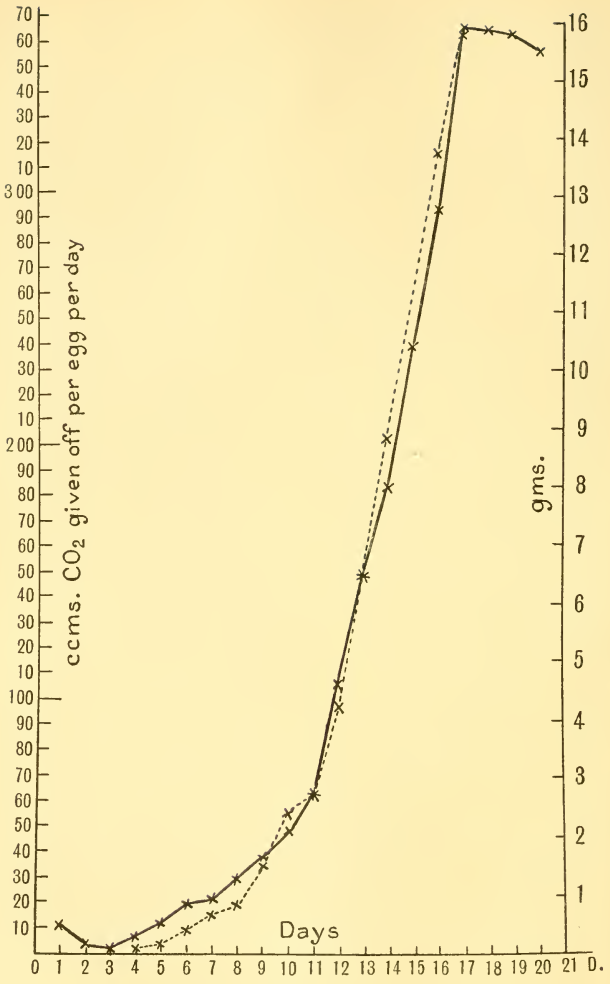


Fig. 141.

—— $CO_2$ ----- weight

practically the adult level (Regnault) by half-way through the incubation period, but Bohr & Hasselbalch's weighings were few in number, and it would not be just to lay much emphasis on the shape of the curve. That it descends so markedly, in opposition to the ascending curves of the earliest stages in echinoderms and amphibia, for example, is all that need at present be stressed.

In the second paper Hasselbalch went on to investigate the oxygen consumption of the eggs, and to calculate the respiratory quotient. This last had only previously been approached by Baumgärtner, who, having found that 1·63 litres of carbon dioxide were given off during the whole of incubation, and that 1·76 litres of oxygen were taken in, concluded that the average respiratory quotient was 0·93. Pott & Preyer's respiratory quotients had been obviously wide of the mark, reaching in some cases 3·59. Hasselbalch devoted a long preamble to the shortcomings of the earlier investigations, and first paid attention to the growth-curve of the chick embryo, adding more figures to those of Bohr & Hasselbalch, which are given in Appendix 1. He made measurements also of the relation between the embryo and its membranes, and of the water-content of embryo and allantois—these are mentioned elsewhere in their proper place.

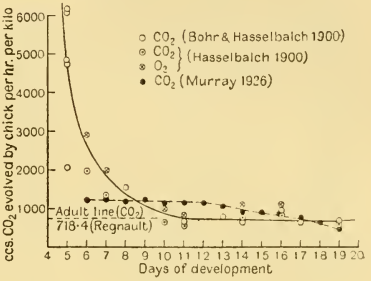


Fig. 142.

Controls on infertile eggs showed that only an extremely small amount of oxygen was taken up by eggs without living embryos, not more than 0·15 c.c. per hour. A slight escape of nitrogen from the eggs seemed to occur, but was very insignificant in amount. Hasselbalch then went on to experiments with fertile eggs. The oxygen consumption per hour followed the carbon dioxide output closely, and rose in the same way as the weight curve, just as Bohr & Hasselbalch had found to be the case for the increments of carbon dioxide production. The respiratory quotients found varied round about 0·7, but discussion of them will be deferred for a moment. Hasselbalch found that, during the entire incubation

period, 3·0225 litres or 5·939 gm. of carbon dioxide were produced, so that, calculating from an average respiratory quotient of 0·677, 4·465 litres or 6·384 gm. of oxygen was used, and that value was, indeed, very close to the one experimentally found. Hasselbalch reckoned that, as the 5·939 gm. of carbon dioxide given off corresponded to 1·620 gm. of carbon, and as, according to Liebermann, egg-fat contained 71·67 per cent. carbon, 1·620 gm. of carbon accounted for 2·260 gm. of fatty acids. This was in very encouraging agreement with Liebermann's figure, calculated from chemical analysis, that 2·762 gm. of fatty acids disappeared during development. It was not unnatural that Hasselbalch should conclude that fatty acids were the sole source of embryonic energy during incubation, though to do so was certainly to forget the presence of protein breakdown-products in the allantoic fluid, so obvious in the egg, and to take insufficient account of the intervention of more complicated processes than the simple oxidation of fatty acids to carbon dioxide and water. From his oxygen figures, Hasselbalch went on to calculate the metabolic rate, which worked out in good confirmation of the rates reported in the previous paper, and is shown in Fig. 143. The weight of the membranes is not included in this calculation, and until some information concerning their respiratory intensity is available, their influence on the metabolic rate curve cannot be assessed (cf. the data of Byerly in Appendix 1).

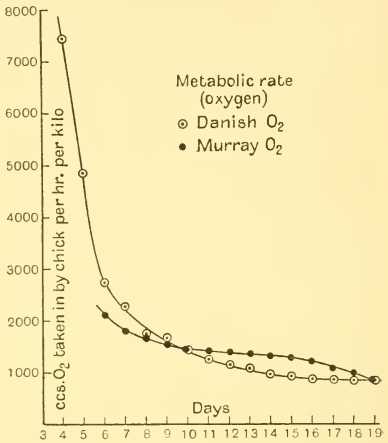


Fig. 143.

Hasselbalch concluded as the result of his experiments that an exceedingly small amount of a gas other than carbon dioxide was lost by the egg during its development, and believed it to be nitrogen. He returned to this puzzling phenomenon in a subsequent paper in which he again asserted its objectiveness, maintaining that 0·5 c.c.

of oxygen and 2·0 c.c. of nitrogen were given off by fertile eggs during the first week of incubation. He tried many methods in his efforts to get to the bottom of it; thus he studied the gaseous exchange of the whole yolk *in vitro*, the composition of the air in the air-space, and of that extractable from eggs by bringing them into a high vacuum. His results left him with the conviction that there was an output both of oxygen and nitrogen, and he suggested various possible mechanisms which would account for it. Typical experiments are shown in Table 81. It is probable that they simply represent the

Table 81. *Hasselbalch's experiments.*

| | Time of the experiment in hours | Cubic centimetres of gas | | | | | |
|---|---|---|---|---|---|---|---|
| | | Carbon dioxide | | Nitrogen | | Oxygen | |
| | | Taken up | Given off | Taken up | Given off | Taken up | Given off |
| Fertilised egg, 1st day | 5·0 | — | 0·09 | — | 0·36 | — | 0·44 |
| ,, ,, | 5·0 | — | 0·15 | — | 0·78 | — | 0·24 |
| ,, ,, | 5·0 | — | 0·05 | — | 1·40 | — | 0·40 |
| Fertilised egg, 2nd day | 4·5 | — | 0·04 | — | 0·10 | 0·04 | — |
| Unfertilised egg | 5·5 | 0·34 | — | — | 1·47 | — | 0·85 |
| ,, | 4·0 | 0·02 | — | — | 0·88 | — | 0·39 |
| ,, | 4·5 | — | 0·37 | — | 2·37 | — | 0·61 |
| ,, | 4·0 | — | 0·09 | — | 1·06 | — | 0·35 |
| Fertilised yolk in 0·82 % saline | 4·5 | — | 0·06 | — | 0·13 | — | 0·16 |
| ,, ,, | 6·25 | 0·10 | — | — | 0·11 | — | 0·13 |
| ,, ,, | 4·0 | None | None | — | 0·34 | — | 0·20 |
| Fertilised yolk in 0·59 % sodium fluoride | 4·5 | — | 0·13 | — | None | — | 0·13 |
| Unfertilised yolk in 0·82 % saline | 4·0 | 0·05 | — | 0·01 | — | — | 0·06 |
| ,, ,, | 6·0 | — | 0·10 | 0·16 | — | — | 0·06 |

adjustment of the shell, the yolk, and the white, as they gain gaseous equilibrium with the external air. Hasselbalch unfortunately did not state how long a time elapsed between the laying of the eggs and his respiratory experiments upon them. In spite of the fact—plain from his tables—that unfertilised eggs gave off as much oxygen as fertilised ones, he persisted in maintaining that "the condition for physiological oxygen-production in the first hours of development is the presence of living cells". By means of his *in vitro* experiments with yolks in salt solutions he showed that the osmotic pressure had an influence on the gases given off; thus hypotonic solutions (1 per cent. sodium fluoride, etc.) led to a decrease in the oxygen generated, but hypertonic solutions set up oxidation processes in the yolk which abolished it altogether by causing an oxygen uptake.

"The oxygen may be", said Hasselbalch, "either a by-product of syntheses associated with cell-division—possibly of fundamental nature, analogous to the $O_2$-assimilation of green plants, and normally obscured by the mass of respiration—or on the other hand, it may be purely a side-issue, originating from some fermentative process or other associated with growth." And there is, as we have seen, a third possibility.

Brandes has more recently discussed the question afresh and regards the oxygen given off by the yolk in Hasselbalch's experiments as of much importance for the life of the embryo, cut off as it is from the air by the thick shell and the mass of albumen. His remarks do not include any chemical explanation of its origin but as we shall see in Section 14·6 the yolk of the avian egg contains catalase in an active condition and it is legitimate to suppose that the concentration of hydrogen peroxide, activity of the enzyme, etc., may be so arranged that an appreciable part, if not all, of the oxygen requirements of the embryo during the first day or two, is provided for in this manner. Brandes divides the respiratory life of the chick embryo into the following stages:

A. A "molecular" respiration of yolk-oxygen
   (i) without the presence of haemoglobin (1 day),
   (ii) with the presence of haemoglobin (2nd to 19th day),
      (a) by the blastoderm circulation (up to the 6th day),
      (b) by the yolk-sac circulation (up to the 19th day).

B. A respiration of atmospheric air
   (i) through the allantoic vessels (from the 5th day onward),
   (ii) through the lungs (from the 17th day onward).

Brandes called attention to the old work of Dulk in this connection. It is doubtful whether stress can be laid on the results obtained with the technique of 1830, but nevertheless it is interesting to recall that the gas which Dulk obtained from whole eggs contained 6 per cent. more oxygen than atmospheric air and, after 10 days' development, $1\frac{1}{2}$ per cent. Dulk's work has already been referred to on p. 617. His figures were:

| | % oxygen |
|---|---|
| Air ... ... ... ... | 20·8 –21·1 |
| Gas from whole eggs (0 days) | 25·26–26·77 |
| ,, ,, (10 days) | 22·47 (with 4·44 % carbon dioxide) |
| ,, ,, (20 days) | 17·9 (with 8·48 % carbon dioxide) |

In one respect, at any rate, Hasselbalch's experimental findings were subsequently reversed, for a few years later Krogh, who was undertaking an extended test of the question whether animals excrete small amounts of nitrogen or not, took occasion to examine the hen's egg from this point of view. His experiments, which were conducted with irreproachable technique, led to quite negative conclusions. For the most part he occupied himself with later stages than Hasselbalch, but even on the 1st day of development he could find no evolution of nitrogen which was outside the small experimental error. His conclusion was that certainly not more than 0·003 c.c. per hour, or 2·5 mgm. of nitrogen for the whole 3 weeks of development, was excreted, and so far the 2·5 mgm., if indeed they do leave the egg, have not been missed by chemists. Krogh's experiments were fully supported by some work of Tangl & von Mituch. "Hasselbalch found", said Krogh, "by evacuating egg-contents in the mercury pump, that fresh eggs contain a considerable surplus of dissolved gases above that which could be taken up by a corresponding quantity of pure water. The surpluses are, according to him, confined to the yolk and I venture to suggest that it is the fatty substances which dissolve the gases. In an egg of 60 c.c. about 1·2 c.c. of nitrogen is contained whereas 60 c.c. of water saturated with air at 38° contain only 0·55 c.c. Hasselbalch found that after two days' incubation the surplus had sensibly diminished and there can be no doubt that the whole of it will be given off during development as the substances of the chicken dissolve less of the gas than pure water."

Pott had claimed that much more carbon dioxide was given off by an egg in pure oxygen than in ordinary air, but his technique was inferior. Hasselbalch found that whatever the effect was, it was very variable; thus in one experiment in 82 per cent. oxygen the carbon dioxide output was half the normal, and the oxygen uptake only a quarter the normal, but in 79 per cent. oxygen the carbon dioxide output was slightly raised, while the oxygen uptake was three times the normal. These effects could only be due to toxic action of high oxygen concentrations (see Riddle's work in Section 18·9) alternating with true accelerating effects. Hasselbalch also concluded that the excretion of gaseous nitrogen from the eggs could take place at these high oxygen concentrations to a far greater extent than under normal conditions, as much as 2·268 c.c. being given off per hour in one experiment. Krogh's work, which did not

include data for abnormal oxygen concentrations, cannot help us here, and Hasselbalch's observations on nitrogen excretion still remain mysterious.

The observations of Bohr & Hasselbalch on the heat production of the hen's egg were published in 1903. They knew the amount of oxygen used and carbon dioxide excreted, and the amount of fat

Table 82. *Respiratory quotients of Chick.*

| Days' development | Given by Lussana | Given by Bohr & Hasselbalch | Calculated by Needham from the figures of Bohr & Hasselbalch | Given by Hasselbalch | Given by Murray |
|---|---|---|---|---|---|
| 1 | — | — | — | 0·642 | — |
| 2 | — | — | 1·010 | 1·318 | — |
| 3 | — | — | 0·960 | 0·341 | — |
| 4 | — | — | 1·141<br>0·697 | 0·801 | — |
| 5 | — | 0·890 | — | 0·673 | — |
| 6 | — | — | 0·619 | 0·653 | 0·60 |
| 7 | — | — | 0·560<br>0·600 | 0·606 | 0·69 |
| 8 | — | 0·655 | 0·530<br>0·500 | 0·710 | 0·75 |
| 9 | — | 0·733<br>0·689 | 0·500 | 0·679 | 0·79 |
| 10 | — | 0·747<br>0·703 | 0·500 | 0·706 | 0·81 |
| 11 | — | — | 0·490<br>0·450 | 0·734 | 0·82 |
| 12 | — | 0·751 | 0·300 | 0·628 | 0·81 |
| 13 | — | 0·646<br>0·712 | 1·000 | 0·685 | 0·79 |
| 14 | 0·533 | 0·705<br>0·675 | — | 0·669 | 0·76 |
| 15 | 0·527 | 0·681 | 0·602 | 0·647 | 0·72 |
| 16 | 0·514 | 0·735<br>0·679 | — | 0·716 | 0·70 |
| 17 | 0·614 | 0·708 | — | 0·678 | 0·69 |
| 18 | 0·630 | 0·718 | — | 0·657 | 0·70 |
| 19 | 0·761 | 0·693 | — | 0·716 | 0·71 |
| 20 | — | — | — | 0·675 | — |
| 21 | 0·680 | — | — | — | — |

burned. They rightly regarded it as very important to know whether the energy of the fatty acids could all be accounted for as heat put out during development, and, if not, what proportion of it could be. With this aim in view they constructed a differential calorimeter in which they were able to incubate single eggs and examine the heat production of them at the same time as their gaseous exchange. The figures they obtained were very numerous. Attention may first be directed to the respiratory quotients, which are collected together in Table 82 and in Fig. 144. Five series are available, those of Bohr

& Hasselbalch, those of Hasselbalch, those calculated from Bohr & Hasselbalch's data, those of Lussana, and those of Murray, whose papers will be mentioned below. As Fig. 144 shows, there are a certain number of points above 0·75, although the greater number lie just below that figure. What is probably significant is that the latter occur mostly after the 8th day of development, while the

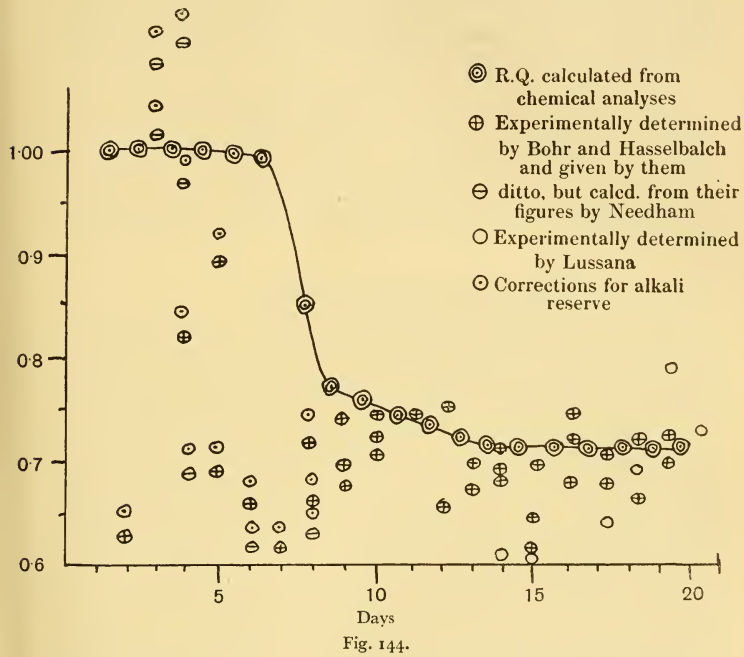


Fig. 144.

former all occur before it. No high point is to be found after the 10th day[1]. Lussana's points agree well with those of Bohr & Hasselbalch and Hasselbalch. Of the high respiratory quotients, the only point actually given by Bohr & Hasselbalch was 0·890 for the 4th day; the others were all calculated from their data in 1927 by me. At the same time I also attempted to see to what extent the alkali

[1] Dickens & Simer give the R.Q. of 5th day chick embryos *in vitro* (phosphate-Ringer, *p*H 7·4, 38°, with 0·2 % glucose) as unity.

reserve of the egg-contents would affect the values obtained for gaseous exchange, for this source of error was neglected altogether by the Danish workers. From the careful investigations of Aggazzotti on the *p*H, and of Healy & Peter on the total acidity of the yolk and white, I made an estimate of the extent to which the respiratory quotient values would be affected if carbon dioxide were retained and neutralised instead of being excreted. The results, shown in Fig. 144 by special points, showed that the values might come higher by as much as 0·5 respiratory quotient units when so corrected, but not more. The effect of the alkali reserve may therefore be regarded as quite small. The line joining the series of points in Fig. 144 represents the respiratory quotient calculated from various chemical analyses; this will be referred to in detail later. On the whole, the evidence points to a combustion of fat after the 8th day (Bohr & Hasselbalch's average then was 0·68) and to more complicated events before that time.

### 4·12. Heat-production of Avian Embryos

Bohr & Hasselbalch's observations on the heat-production of the egg are shown graphically in Fig. 145. There are several remarkable things about this curve. Firstly, during the first few days of development they observed an absorption of heat, not an output. They were convinced that this process could not be accounted for as a meaningless effect due to technique, but that it was a real physiological phenomenon, and they associated it with the production of oxygen which Hasselbalch had previously shown to go on before the 3rd day. They pictured the existence of some endothermic synthetic process which gave off oxygen as a by-product, and even suggested that this might go on throughout development obscured by the mass of the usual respiratory exchange. No satisfactory explanation has so far been advanced for the initial heat absorption shown in Bohr & Hasselbalch's work, and, as no calorimetry of the egg has since been published, it has remained unconfirmed, although Barott, I understand, has unpublished experiments indicating that it is an artifact. Its possible theoretical importance has already been indicated in connection with the work of Rapkine on the echinoderm egg (see p. 648). The second striking thing about the figure is the fact that the observed values for heat-production agree so well with the values calculated from the oxygen taken in and the carbon dioxide

put out, on the assumption that the combustion is going on at the expense of fat. The observed and calculated points lie for the most part on exactly the same line. Table 83, which gives the average values for observed and calculated calories evolved per hour on each day, shows up the divergences better. After the early period of heat absorption, there follows a second period in which the observed values are somewhat lower than the calculated ones but by the 11th day equivalence has been gained, and, in the third period,

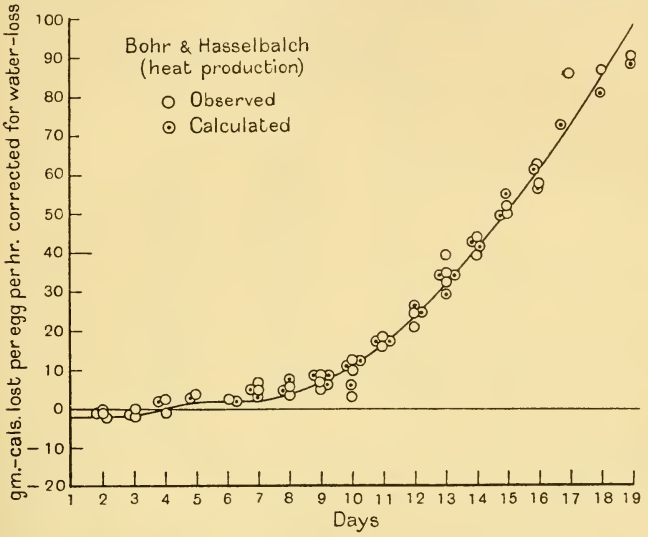


Fig. 145.

when the heat radiated is reaching high figures, the advantage is rather on the side of the observed values. Bohr & Hasselbalch noticed this fact, and, by a comparison of the percentage differences in each case, showed that they cancelled out almost exactly over the whole time of incubation, the earlier lag on the observed side making up for the later lag on the calculated side. Thus for the entire incubation period 12·16 kilo cal. were experimentally found in the calorimeter, while 12·11 kilo cal. were calculated as the expected amount —an excellent agreement. The extreme importance of these experi-

ments will appear in the section on Energetics and Energy Sources of the embryo. For hourly radiation the correspondence was equally good, being 506·8 gm. cal. observed and 504·72 calculated. Thus there was no energy unaccounted for, none held back for the purpose of maintaining the embryo in physico-chemical equilibrium, or as "Entwicklungsarbeit". "Während der Entwicklung des Embryos", as Bohr & Hasselbalch put it, "in bedeutenden Mengen

Table 83. *Heat-production of chick embryo in gram calories per hour produced.*

| | Averages | | |
|---|---|---|---|
| | Observed | | Calculated from the respiratory exchange |
| Days | Original data | Smoothed | |
| 1 | — | — | — |
| 2 | −0·77 | −1·50 | — |
| 3 | −1·47 | −0·90 | — |
| 4 | 0·39 | 0·39 | 2·52 |
| 5 | 3·40 | 2·00 | 2·84 |
| 6 | 2·63 | 3·22 | 3·75 |
| 7 | 5·11 | 4·55 | 5·86 |
| 8 | 4·23 | 5·27 | 6·62 |
| 9 | 7·16 | 6·69 | 8·56 |
| 10 | 8·42 | 10·19 | 10·20 |
| 11 | 17·27 | 17·10 | 17·77 |
| 12 | 24·63 | 24·63 | 24·85 |
| 13 | 35·38 | 32·50 | 32·76 |
| 14 | 41·62 | 41·62 | 42·97 |
| 15 | 51·64 | 51·64 | 53·24 |
| 16 | 60·14 | 61·50 | 59·48 |
| 17 | 86·02 | 73·00 | 73·18 |
| 18 | 87·06 | 82·50 | 81·45 |
| 19 | 90·07 | 90·07 | 89·75 |

umgesetzten chemischen Energie auf neugebildete Gewebe nichts übergeführt wird, dass dieselbe dagegen in ihrer Gesammtheit das Ei als Wärme verlässt." Tangl's earlier papers were appearing at this time, and they naturally found in his estimations of the calorific value of the egg-substance at different times during development confirmation of their view that fatty acids were the exclusive source of energy by combustion. It is only fair to add that they did take into account the possibility of combustion of proteins, but their arguments against it were extremely poor. From their figures for heat-production, Bohr & Hasselbalch did not calculate the metabolic rate by referring them to 1 gm. of embryonic body-weight, probably because they were doubtful about how much the membranes might be producing. The results of such a calculation[1] are shown in Fig. 146,

[1] Leaving the membranes out of account.

from which it can be seen that the fall is most pronounced, the metabolic rate being above 15 gm. cal. per gram on the 5th day and below 4 gm. cal. per gram on the 19th day. The significance of the kink in the curve at the 9th day is obscure. As the curve in which Murray's weight measurements are used shows it too, it must be due to the heat data. On the whole, a good resemblance can be traced between the heat-production metabolic rate curve and the gaseous exchange metabolic rate curves shown in Fig. 143, for in both cases the low value characteristic of the end part of incubation is attained about half-way through development or slightly before. The question of relating the heat-production to the surface of the chick embryo, which is rather a complicated one, will be left until the section on Energetics. If now, aided by the investigations of Bohr & Hasselbalch, we enquire what are the number of gram calories produced during each period of weight doubling, we find there is first a fall and then a rise. In Fig. 147, which has been plotted from such a calculation, the times required to double the weight at different ages are given along one ordinate, while the gram calories per gram evolved during the periods of doubling are given along the other. This curve does not at all resemble that found by Gayda for the frog, in which there was a continual rise, and from

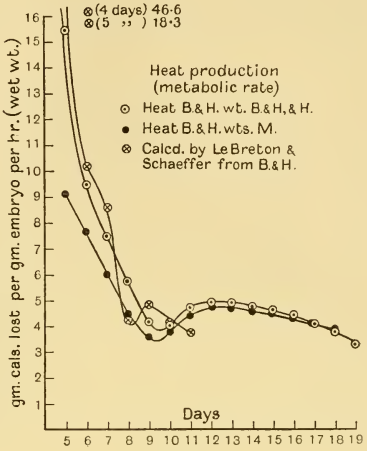


Fig. 146.

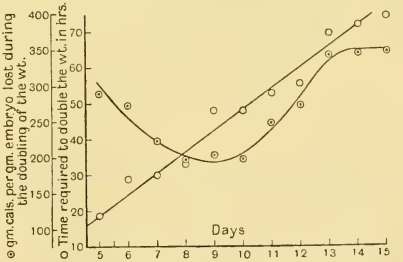


Fig. 147.

the trough in it the conclusion might be drawn that a point of maximum efficiency was passed through at the 9th day. But it must be remembered that this curve is the result of many contributing sets of figures, and depends on various assumptions which will be discussed later.

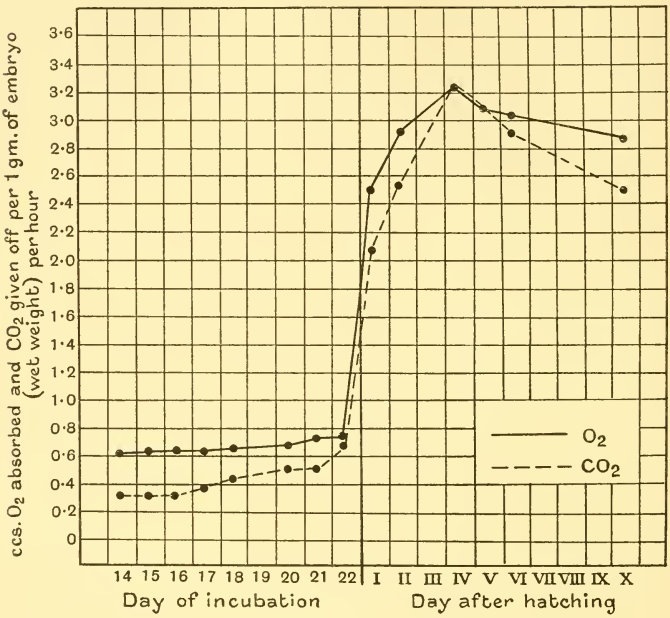


Fig. 148.

## 4·13. Later Work on the Chick's Respiratory Exchange

Lussana's paper was not devoted solely to the respiration of the chick in the egg. His experiments did not begin till the 14th day of development, but their special interest is that he carried them through the hatching process and for some days afterwards. Fig. 148 taken from his paper shows his results for metabolic rate. Beginning at a more or less constant level, just as had been found by the Danish workers, it rose very sharply indeed at hatching, and re-

mained some five times greater as far as Lussana followed it. Here, of course, the effects of muscular movement are coming into play, and Lussana's figure reminds us that, towards the end of embryonic life, the concept of "basal" metabolism becomes important. During the early stages, the embryo can be considered as under basal conditions, except for the fact that it is constantly absorbing nourishment, because the factor of muscular activity is not operating. The metabolic rate of the echinoderm gastrula, for example, needs no

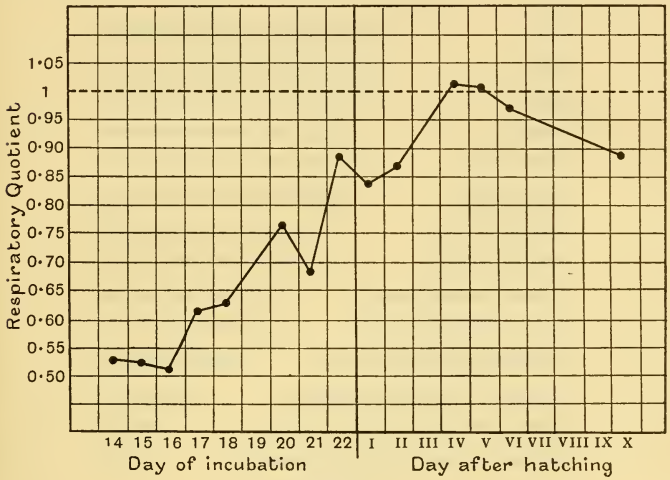


Fig. 149.

such correction. In any case it is not easy to say whether the general notion of basal metabolism can be applied to an embryo at all, and caution must certainly be used with respect to late stages where increasing cardiac activity and muscular movements in the amniotic cavity may affect the readings. A fact which obviously confirms these interpretations of Lussana's figures is that, during the hatching process, the respiratory quotient steadily rose until it reached unity 3 days after the chick had come out. Fig. 149, taken from Lussana, demonstrates this, and shows the transition from the characteristic fatty respiratory quotients of the last week of incubation to the state of affairs in which much carbohydrate is being used for

muscular work. Lussana himself thought that the new environment into which the chick enters at hatching had perhaps a stimulatory effect on all the organs of the body, and so led to increased carbohydrate utilisation.

Investigations of the same type as those of Bohr & Hasselbalch were afterwards carried out by Lamson & Edmond, by Atwood &

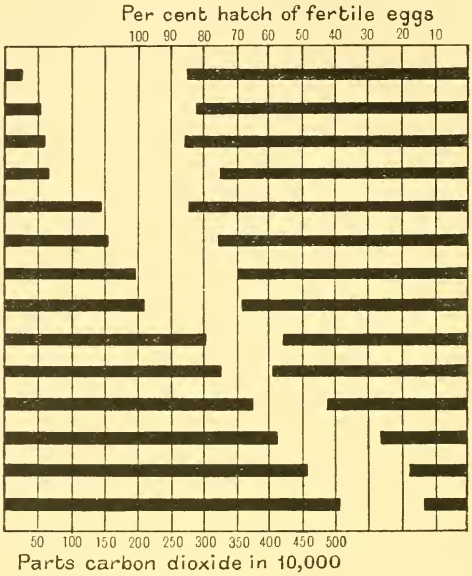


Fig. 150.

Weakley, by Murray and by Hanan. Lamson & Edmond's work was mostly concerned with the efficiency of incubators from a respiratory point of view, and they dealt with the optimum concentration of carbon dioxide in the circulating air. They also obtained some interesting results by analysing the air under sitting hens, leading off samples for analysis by means of a perforated dummy egg connected to a pipe. They found that the air under sitting hens does not get much richer in carbon dioxide if china eggs

are used, but does if the eggs are fertile and developing properly. Other factors which may increase it are the respiration of the hen and the evolution of carbon dioxide from the soil. It never reached a toxic concentration, however, although usually well above the ordinary air of the room.

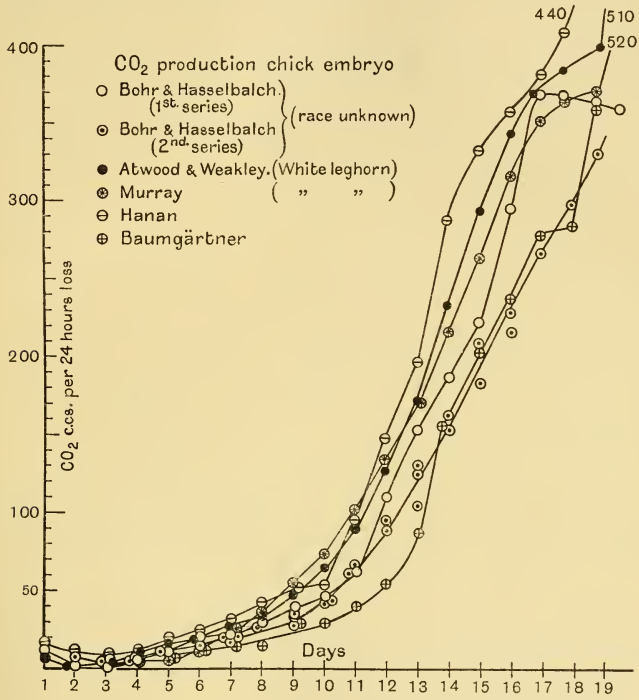


Fig. 151.

Uncertainty about the action of carbon dioxide on development, whether good or bad, had previously led to a good deal of work designed to discover what proportion of the gas in the circulating air gave the best results. Lourdel; Brigham; Thom and Lamson & Kirkpatrick advocated the addition of carbon dioxide to the air, and Dryden; Edmond and Harcourt & Graham considered that

greater ventilation should be used, as too much was provided by the embryo itself. Lamson & Edmond, using incubators of identical form and a very large number of eggs, simply varied the ventilation, the output of carbon dioxide from the eggs being sufficient as a source. The results they obtained are best shown in the diagram given in Fig. 150. The percentage hatch was never more than 85, and seemed to be unaffected by the amount of carbon dioxide present if it does not exceed 150 parts per 10,000; above that figure, however, the hatch is much decreased, and when 500 parts is reached falls as low as 14. Subsidiary experiments with added carbon dioxide fully confirmed the results given. Rogalski has since studied the details of this effect of carbon dioxide on chick embryos. Lamson & Edmond were of opinion that their tests demonstrated diffusion rather than secretion of gases to take place through the allantoic membrane in the egg, for, when the carbon dioxide in the air was increased considerably, that provided by the embryos slightly decreased (ratio of 4·27 instead of 4·46). They also confirmed the close relation found to hold by Bohr & Hasselbalch between the weight growth curve and the increment curve of carbon dioxide production. (Their weight figures are given in Appendix 1.)

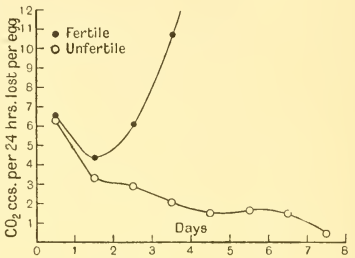


Fig. 152.

More directly physiological investigations were those of Atwood & Weakley, whose values for carbon dioxide production in cubic centimetres per egg per day are shown in Fig. 151. The technique of these workers was very good, probably better than any of the others. They regarded the ideal conditions to be 50 parts of $CO_2$ per 10,000, and gave a table showing the amount of ventilation in cubic feet of air per 100 eggs per day necessary to maintain the concentration of the gas at that level. In agreement with Bohr & Hasselbalch, they found an initial output of carbon dioxide which was certainly not coming from the embryo, but from the shell and the contents[1]. Fig. 152, in which are placed some of their values for fertile and infertile eggs respectively, demonstrates the kind of result they ob-

[1] See also p. 819 on this subject.

tained. Brody has discussed this phenomenon, which, of course, appears very obtrusive when carbon dioxide output is calculated like percentage growth-rate. They found it relatively easy to tell whether the embryo had died, for in such cases an obvious falling away from the rising curve appeared. Benjamin showed that, other things being equal, the larger the egg the larger and more vigorous

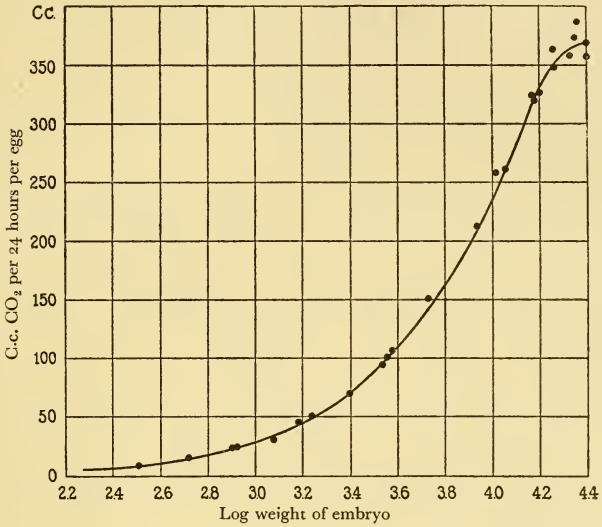


Fig. 153.

the chick hatched therefrom (contradiction with Riddle's results on pigeons (?); see p. 254), so Atwood & Weakley arranged their results on carbon dioxide in a correlation table. They found that the correlation was positive and significant, i.e. the larger eggs tend to give off more carbon dioxide during the whole period than the smaller ones. This would by no means have followed *a priori*.

The most recent example of this kind of experiment is the work of Murray. He only used eggs which had been allowed to come into gaseous equilibrium with the air by standing in the apparatus for some time before the experiment began, so he was able to leave out

of consideration the early production of carbon dioxide by the shell. His experiments differed from those of his predecessors in that he made no attempt to incubate the eggs in the respiration apparatus, but studied their carbon dioxide output for short periods removed from the incubator, from the 6th day onwards. Having obtained a final set of figures, he related the cubic centimetres of carbon dioxide put out per 24 hours to the log. weight (as shown in Fig. 153), and then, reading off on the ordinate the amounts of carbon dioxide corresponding to the log. weights at definite days, obtained a graph relating carbon dioxide production to age. This is naturally more accurate than proceeding directly. The curve so obtained is shown in Fig. 151, together with those of Bohr & Hasselbalch, of Atwood & Weakley, and of Hanan. Taking the data as a whole, it is evident that, for the most part, the values of the American workers are higher than those of the Danish ones; this is probably a difference of breed.

The beginnings and the ends of the curves are interesting. All the curves which go as far back as the 1st day show the small output of carbon dioxide from the shell, and all of them, except the second series of Bohr & Hasselbalch, show the falling off about the 17th day, which appears also in the sigmoid form of Murray's carbon dioxide per log. weight curve just given. For an explanation of this phenomenon, see Brody (p. 431). Murray's curve begins slightly below the Danish ones, then between the 7th and 17th days moves up above them, suffering a temporary eclipse on the 18th day. Murray did not offer any explanation for these small divergences. In a later paper he calculated the metabolic rate from these carbon dioxide figures, and in Fig. 154 is shown the cubic centimetres of carbon dioxide produced per gram embryo (wet weight) per day plotted against the age from 5 to 19 days of incubation. The Minot growth-rate curve is included to illustrate the diphasic character of the whole process (this has already been discussed on p. 547). It is evident that the metabolic rate declines with age, slowly before the 11th day and afterwards much faster. If this graph be compared with that already given for metabolic rate as calculated from the gas and weight values of the Danish school (Fig. 142), it will be seen that, although the metabolic rate in their case certainly declines, it does so in harmony with the (Minot) growth-rate, being relatively rapid at the beginning and relatively slow at the end. It therefore does not obey Murray's rule. At present no explanation seems avail-

able for this, but Fig. 142 shows that, as far as absolute values go, Murray's curve corresponds with the slowly falling part of Bohr & Hasselbalch's. Perhaps if Murray had ventured into the region before the 6th day, he would have got higher points, and would have

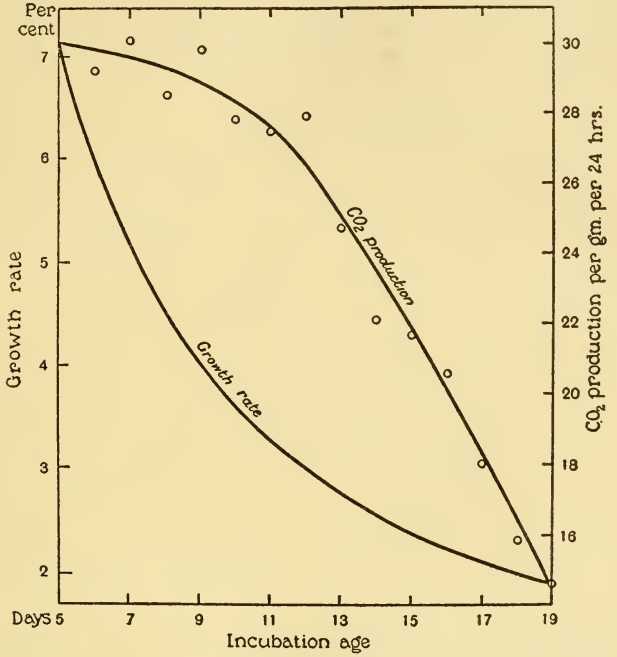


Fig. 154.

had to draw an S-shaped curve, as afterwards happened in the case of oxygen.

The only attempt that has been made to influence the metabolic rate in the embryo is the interesting one of Hanan, who in 1928 injected doses of about 1/30,000 to 1/40,000 mgm. of thyroxin in 0·25 c.c. of dilute alkali into the air-spaces of developing eggs on the 7th day of incubation. For about 5 days the injected eggs

consumed rather more oxygen and gave off rather more carbon dioxide than the controls, but after that exactly the reverse relation held good, the cross-over taking place on the 12th day (see Fig. 155). As has already been mentioned (p. 539), the weights of the embryos from the injected eggs were always within minute limits the same as those from the controls. It is therefore evident that the metabolic rate must have been intensified during the first period, and diminished afterwards. Evidently in Hanan's experiments the developmental machinery was dissociated into its components, metabolic rate being affected but not growth-rate.

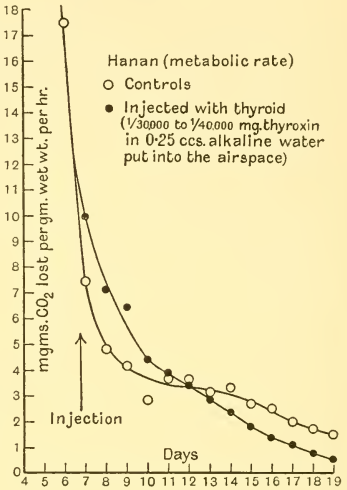


Fig. 155.

In a later paper, Murray went on to study the oxygen intake of the developing egg by accurate methods involving the use of a differential manometer in which convection currents were set up by leading thin-walled rubber tubes containing cold water past the vessel and in contact with it. Murray neglected to give the figures he obtained for cubic centimetres of oxygen used per egg per day, and only published the metabolic rate, but the former can be calculated. In Fig. 156 they are shown, so that here also the American values come higher than the Danish ones. Murray's metabolic rate for oxygen appears in Fig. 143, where the cubic centimetres of oxygen used per gram (wet weight) of embryo per day are plotted against the age from 5 to 19 days. Again, there is a notable decline, but the curve, as drawn, does not follow the carbon dioxide values very well, and presents an S-shaped appearance instead of being concave to the abscissa, as Murray's rule requires. However, the burden of the highest part of the curve rests on one point only, and, if that were wrong, the appearance of the whole would resemble that of the carbon dioxide metabolic rate curve very closely. Murray himself

was inclined to regard the oxygen curve as the more reliable, for much the same reasons as have already been brought forward in the case of echinoderm work. The uncertainty of alkali reserve, and the known utilisation of carbon dioxide in the transport of calcium from shell to bones, obviously introduce doubtful factors. The total oxygen consumption for the first 19 days estimated by graphical integration came to 2988 c.c., which, on the basis that fat only is burned, leads to the conclusion that 1·48 gm. of dry substance is oxidised during that period. If only protein and carbohydrate were burned, it would require over 3·28 gm. to use the observed amount of oxygen. Chemical analyses, to be discussed later, show, however, that approximately 1·62 gm. of solid substance is burnt during the first 19 days, a figure which can be accounted for on the assumption that 92 per cent. of the catabolism is oxidation of fat, and the rest of protein and carbohydrate. Murray's work on carbon dioxide output had led him to assess the amount of fat burned as 98 per cent. of the total food-stuff catabolised, but that figure is certainly too high. In direct bearing upon these questions was his calculation of the respiratory quotient. According to him, it varies during the period under discussion from 0·82 to 0·60. Curiously enough, his lowest value, 0·60, he obtained on the 6th day, i.e. the very time when a particularly high one would have been expected. However, if his oxygen consumption on that day was in error on the high side, as has already been surmised, then the true respiratory quotient for that moment would be much higher.

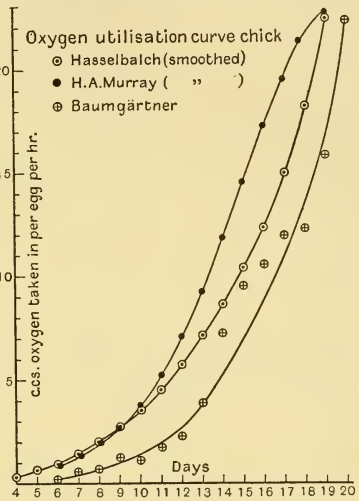


Fig. 156.

Apart from the work of Warburg and his collaborators, which will be treated as a whole below, Shearer is the only investigator who has

examined the *in vitro* respiration of chick embryo tissues. His experiments have already been discussed in relation to Child's theory of metabolic gradients. If reference be made to Fig. 96, it will be seen that the work led to two conclusions, firstly, that the respiratory rate of the head fragments was greater than that of those from the tail, and

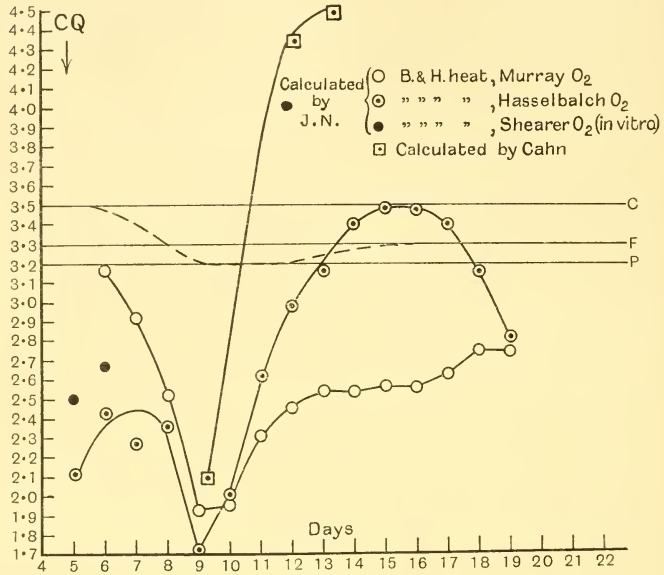


Fig. 157.

secondly, that in both cases the rate declined as development proceeded. Reasons have already been given for doubting the significance of the first of these results, but the second remains unaffected, and adds another piece of evidence to the well-established belief that in the chick embryo metabolic rate declines with age. An amount of tissue containing 2·8 mgm. of nitrogen takes up oxygen as follows:

| Day | Oxygen in c.mm. |
|---|---|
| 4 | 18·50 |
| 5 | 12·50 |
| 6 | 5·80 |
| 7 | 3·02 |
| 10 | 1·05 |

Two attempts have been made to calculate calorific quotients for the chick embryo since the publication of exact data for the heat production and the oxygen consumption. The results of my attempt to do this are shown in Fig. 157, where calorific quotient is shown plotted against the age (*a*) using Bohr & Hasselbalch for heat and Hasselbalch for oxygen, and (*b*) using Bohr & Hasselbalch for heat and Murray for oxygen. In both cases there is a fall to the 9th day followed by a rise lasting approximately for the rest of the incubation period, but a glance shows that the values are grossly removed from the theoretical. The dotted line drawn between the horizontal lines shows the course that would theoretically be taken by the calorific quotient supposing that carbohydrate was first burned, then protein, and finally fat. As can be seen, the experimental curves do more or less follow that rhythm, but little weight can be attached to such a correspondence in view of the kink on the heat-production curve (see Fig. 146), which almost certainly is responsible for the drop in the calorific quotient at the 9th day. Nothing would be more welcome than a redetermination of the heat-production curve of the chick embryo, for we should not only then have a better idea whether the kink in question is real or not, but also with improved calorimetry less heat would be lost and the calorific quotient would probably fall within its proper limits. Cahn's views on these questions are discussed in the section on the Energetics and Energy Sources of the embryo.

## 4·14. The Air-space and the Shell

No mention has so far been made of the recent work on the air-space, a structure present in many kinds of eggs, but occupying a specially prominent position in the case of the chick. Its origin in birds' eggs is obscure, but, according to Lataste, no air-space is present before laying, and its appearance is only due to the contraction of the egg-contents from the rigid shell as the egg cools after leaving the parent body. Lataste supports this view by adducing the fact that eggs with flexible coverings never have air-spaces (e.g. lizards and serpents), and, in the case of shell-less birds' eggs, which are sometimes laid, no air-space appears, though the envelopes may be a little wrinkled. Indeed, as long ago as 1847, Coste had observed that if a laying hen was killed, the oviduct ligated, removed, and then immersed in a dish of oil, an oil-space formed

instead of an air-space as the egg and the oviduct slowly cooled. And by clipping a window in the uterine wall, he could produce it at will at any part of the egg. The analogous air-space in the cocoon of the silkworm has been studied by Portier & de Rorthays, who found that carbon dioxide accumulates in it as metamorphosis proceeds, just as during development in the hen's egg. Dubois and Dubois & Couvreur have also studied this air-space.

In the course of his respiration experiments, Hasselbalch investigated the contents of the air-space, obtaining figures as follows:

| Days after fertilisation | C.c. of air in air-space | Oxygen % |
|---|---|---|
| 2 | 1·25 | 20·96 |
| 5 | 0·702 | 21·35 |
| ,, | 0·703 | 21·53 |
| 8 | 0·479 | 21·57 |
| 10 | 1·949 | 20·65 |
| | Average ... | 21·21 |
| Infertile | 0·32 | 21·37 |
| ,, | 0·651 | 20·74 |
| | Average ... | 20·96 |
| | Atmospheric air ... | 20·96 |

Hasselbalch was naturally very interested that the fertile eggs seemed to have slightly more oxygen in their air-spaces than ordinary air, in view of his other researches on the oxygen production of the birds' egg during the first few days. The fact that he got normal figures for infertile eggs still further contributed to that conclusion. But the classical work on the subject is that of Aggazzotti, who in 1914 measured the percentage of oxygen and carbon dioxide in the air-spaces of incubating eggs, not only at sea level but at the mountain experimental station of Col d'Olen.

Taking first the normal figures, Aggazzotti found that in fresh eggs the carbon dioxide content of the air-space is high, from 1·42 to 2·05 per cent., while the oxygen content is just equivalent to that of the external air, though it may be very slightly above it (20·72 to 21·29 per cent.). The former fact agrees excellently with the preliminary output of carbon dioxide (due to the oviduct), which so many workers have observed, and perhaps the latter fact may be taken as confirmation of Hasselbalch's statements about oxygen production. After 8 or 9 hours, however, the carbon dioxide has fallen to 0·6 per cent., while the oxygen has remained stationary,

and, if the egg is infertile, no further change takes place, even though it be kept for a month or more. These alterations are shown in Fig. 158. If the egg is fertile, the carbon dioxide content then rises during the 1st day to 1·89 per cent., a phenomenon probably due to the effect of heat on the egg-contents. The subsequent course of the curve is shown in Fig. 159. A slight diminution on the 2nd or 3rd day brings the value to a level at which it remains until roughly the 11th day (1·06 to 0·33 per cent.), but after that time it progressively augments until hatching. Clearly it is at about the 11th day that the carbon dioxide produced by the metabolism of the embryo becomes greater per unit time than that which can get away

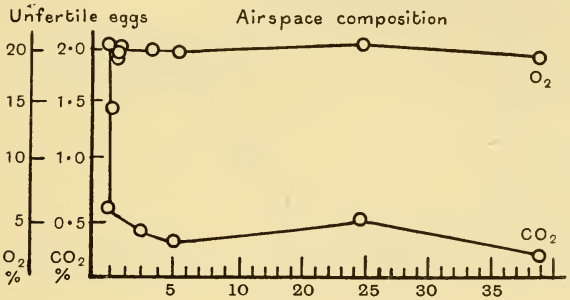


Fig. 158.

through the egg-shell per unit time, hence an increased concentration in the air-space. At the end of development the percentage has risen to between 4·50 and 5·43. During the first week the oxygen content remains unchanged, and a little below that obtained in unincubated eggs, but after that time it begins to fall, presumably because it is being used up by the embryo rather faster than it can diffuse in through the egg-shell. At the end of development it only reaches the figure of 13·65 per cent. If now the curve in Fig. 159 be compared with that found by various workers for the output of carbon dioxide from the egg (Fig. 151), it can easily be seen that, although the latter increases during incubation more than fifty times, the former hardly increases five times; the obvious inference is that the shell becomes more permeable to gases as development proceeds. It is unfortunate that no direct measurements with a diffusiometer

have been made of the shell during the development of the chick, but analyses which will be discussed later (see Section 13·2) do demonstrate that there is a definite loss of inorganic and organic substances from the shell, and it is a well-known fact that the shell becomes more brittle as development proceeds. Only one study of the histology of the shell during incubation has been undertaken, namely, that of Rizzo in 1899. Rizzo found that the number of pores per square millimetre of shell surface varied from 0·86 to 1·44, with an average of 1·23. A hen's egg has an average surface of 6644 sq. mm., and about 7600 pores. Rizzo's method was to drain the contents of the egg

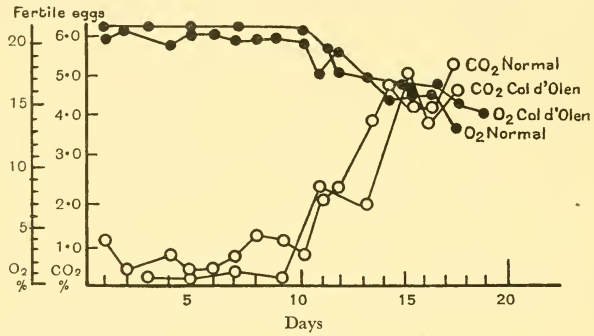


Fig. 159.

through two small punctures, after which the egg-shells were carefully washed, and refilled with a weak aqueous solution of methylene blue —the pores were then visible to the naked eye as fine blue points. They were much more numerous over the air-space than elsewhere[1].

We may conclude that an increased permeability of the shell to carbon dioxide during development is fairly well established and the remarks that have been made on this point apply equally well to oxygen. The loss in oxygen is more or less compensated for by the gain in carbon dioxide, so that the nitrogen content of the air-space remains practically unchanged. Hüfner, whose permeability experiments on egg-shells have already been referred to, found that in 1 second at 11·9° 2·115 c.c. of oxygen would diffuse into the goose's egg (from ordinary air, i.e. a partial pressure of 159 mm.) and 0·503 c.c. of carbon dioxide would diffuse out (to a partial pressure

[1] For a physical account of these pores, see Dumanski & Strukova.

of 29·94 mm.). These findings agree well with those of Aggazzotti. Hüfner himself carried out some experiments on the air-space contents; he was indeed the first worker to contradict the early assertions that there was a much higher oxygen concentration within the air-space than outside. His figures were:

| | % oxygen | % nitrogen | % carbon dioxide |
|---|---|---|---|
| Hen ... ... | 18·94 | 79·97 | 1·09 |
| Goose ... ... | 19·71 | 79·08 | 1·20 |

He made an interesting calculation showing that the amount of the gases which could diffuse in was much in excess of those actually found. Baumgärtner had stated that, on the 20th day of development, a hen's egg gave off 0·56 gm. (285 c.c.) carbon dioxide and took in 0·44 gm. (310 c.c.) of oxygen. Though the surface of the goose's egg is only four times that of the hen's egg, Hüfner allowed a ten times greater metabolism, but even so found that 3100 c.c. of oxygen and 2850 c.c. of carbon dioxide stood much below the 182,700 c.c. of oxygen and the 43,460 c.c. of carbon dioxide which his measurements would regard as being provided by diffusion. However, Baumgärtner's values are very low (60 per cent. of the real values), and the multiplication of those of the hen by 10 to make those of the goose is a hazardous proceeding, so the real gaseous factor of safety of the hen's egg has not yet been calculated, and cannot be until somebody repeats Hüfner's observations on the shell of the hen's egg. From some figures of Hüfner's, however, for experiments in which the inner membrane had been stripped off, a rough assessment of this can be made in the case of the hen. Here in 1 second at 9·4° and from a partial pressure of 159 mm. 1·587 c.c. of oxygen would diffuse into the egg over all its surface (surface values being taken from Murray), and the partial pressure being 29·94 mm., 0·554 c.c. of carbon dioxide would diffuse out. This would mean 137,000 c.c. of oxygen and 47,800 c.c. of carbon dioxide per day. As the greatest amount of oxygen taken in is 720 c.c. per day, and the greatest amount of carbon dioxide put out is 510 c.c. per day, there would appear to be an ample margin. But 137 litres seems an immense quantity of oxygen, and it is probable that Hüfner's figures are here far too high; moreover, the inner shell membranes may probably make a considerable difference.

Another interesting point raised by Hüfner was whether the rates of penetration of gases through the egg-shell followed Graham's law,

and were proportional to the square roots of the specific weights of the respective gases. All his tables showed that they did not follow this rule, but some more complicated one, being doubtless affected by the complex conditions in the material. Hüfner found, as has been said, that hydrogen penetrated most easily, then carbon dioxide, then nitrogen, and lastly oxygen. The hen's egg-shell was less easily penetrable than the goose's egg-shell, and Hüfner suggested that this was necessary for it, since its surface was smaller in proportion to its weight. More recently S. Ancel has shown that the penetration of chloroform vapour into the hen's egg exactly follows Graham's law.

Aggazzotti's experiments with eggs incubated 3000 metres above sea level showed (as may be seen from Fig. 159) that the composition of the air in the air-space was almost identical with the normal sea-level values. The percentage of oxygen and of carbon dioxide was constantly lower by a small amount, so that there was a certain degree of acapnia and anoxaemia of the embryo, due, of course, to the fact that at the Col d'Olen the barometric pressure was only a third of what it was at Turin. These high-level experiments have no great value, for all the embryos incubated at the Col d'Olen died before hatching, and were more or less abnormal.

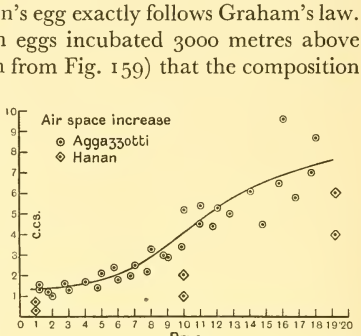

Fig. 160.

No very extensive figures seem to exist in the literature for the change in volume which the air-space undergoes during development, though qualitatively, as is well known, it markedly increases. A curve can, however, be constructed from the data given by Aggazzotti; it is shown in Fig. 160[1]. It will be remembered that the knowledge of this fact was one of the bases of Mayow's theory of embryonic respiration. The elasticity of the air contained in the air-space acted, he thought, like a kind of piston, compressing the yolk and white into the solid tissues of the chick, but we now regard the expansion of the air-space as the effect of development rather than its cause, and as arising from the evaporation of the water and the

[1] Romanov also gives data for this.

combustion of a certain amount of solid. Hanan, in fact, in some unpublished experiments, has noticed a close relation between humidity of environment and size of air-space.

Another interesting corollary of the respiration of the embryo was brought out by Hammett & Zoll. These workers studied the response of the vitelline vessels of the chick embryo to various chemical stimulants, by injecting them into the yolk with a micropipette in the immediate vicinity of a length of vessel observed through a microscope. In this way they ascertained that the walls of the vitelline vessels are sensitive neither to the H ion nor to the OH ion, changes in their concentration varying from *p*H 5·0 to 9·0 provoking no alterations in the calibre of the vessels. On the other hand they are specifically reactive to carbon dioxide and the invariable response was one of constriction—moreover, the effective agent was not $HCO_3$ but either carbonic acid or carbon dioxide, for hydrochloric acid solutions saturated with the gas were as efficient as water saturated with it[1]. What follows, or may follow, from these results, was thus suggested by Hammett & Zoll. It is obvious that temperature variations during incubation under the hen would induce variations in the production of metabolites from which the embryo builds its tissues. With rising temperature this would be accelerated and so would carbon dioxide production. The latter by its constrictive action on the vitelline vessels would cut down the blood-supply to the embryo and thus prevent its being flooded with more food than it could profitably handle. With falling temperature the processes would be reversed, and the relaxed vessels, carrying a larger volume of blood of lesser metabolite content, would thus provide the embryo with adequate material for uninterrupted development. It is conceivable that the regulation of the food-supply is controlled in part by the $CO_2$-sensitivity of the blood-vessels of the yolk-sac. Hammett & Zoll also applied their views to the process of inclusion of the yolk-sac within the embryonic body at the end of incubation, and suggested that the large amounts of carbon dioxide then being evolved constricted the blood-vessels of the yolk-sac to so great an extent as to cause the atrophy which normally occurs.

[1] This was confirmed by Lange. The vascular membranes contain no nerve fibres (Lange, Ehrich & Cohn) and elastic fibres are not to be found in their vessels (Cohn & Lange). The capillaries are more irritable than the arterioles, and at the end of development there is no degeneration; the vessels die in complete possession of their physiological irritability and anatomical integrity.

### 4·15. Respiration of Mammalian Embryos

The data which we have on the subject of the respiration and heat-production of the mammalian embryo are very scanty and fragmentary. The question has been handled usefully from an obstetrical point of view by Harding; Murlin; and Feldman, but a great deal of the information contained in their reviews lies outside the scope of the present book, for it is concerned with changes in the maternal organs during pregnancy. The modern period was opened by Zweifel's discovery in 1876 that oxyhaemoglobin was to be found in the umbilical blood of an infant that had never entered on the pulmonary stage of respiration. This stimulated N. Zuntz to try some experiments in which he asphyxiated the pregnant animal. Most of the work was done by the simple method of ascertaining whether the blood in given blood-vessels was arterial or venous, light or dark, and in this way he found that on asphyxia the foetal circulation would give up oxygen to the placenta and so to the vessels of the uterine wall. Pflüger himself added some remarks to Zuntz's paper, and the line of investigation was continued some years later by Cohnstein & Zuntz in collaboration. Their paper, which was very long, was concerned to a large extent with measurements of blood-volume, enumeration of blood corpuscles, etc., which need only be mentioned briefly here. They were the first to discover that in the earlier stages of development the number of red blood corpuscles in the foetal blood is very low, in certain cases only 1 or 2 being present for every 10 in the maternal blood. A curve constructed from the data of Cohnstein & Zuntz for the growth in the number of erythrocytes is shown in Fig. 440 (Section 17·1).

They also made a good many experiments on the blood-count of newly born infants, and gave in their paper all the literature on that subject before 1884. The early work of Quinquaud; Convert; Wiskemann; and Hoesslin had not succeeded in providing any data on the growth of the haemoglobin-content of the foetal blood, so Cohnstein & Zuntz turned their attention to that, and obtained some interesting results. Discussion of these, however, will be deferred to the section on pigments. Cohnstein & Zuntz also measured the blood-volume in rabbit embryos, and their findings are graphically reproduced in Fig. 161. The volume (expressed as per cent. of the embryo) of the blood in the embryo rises; that of the blood in the placenta

and in the embryo plus placenta falls. Some figures for placenta weight in rabbits are included on the graph. Evidently the placenta circulation fills up with blood before the true embryonic circulation has had time to develop very far, and in the early stages the blood-vessels in the embryo form only a small part of the total placental circulation. All these facts have an obvious importance with relation to foetal respiration. Cohnstein & Zuntz in their paper gave a full bibliography of the earlier work on blood-volume in newly born infants and the young of animals. They also dealt with the changes in the blood following birth, the foetal blood-pressure, the foetal pulse frequency and circulation rate, using Ludwig's Stromuhr. They did not relate any of these determinations very closely with the age of the embryo.

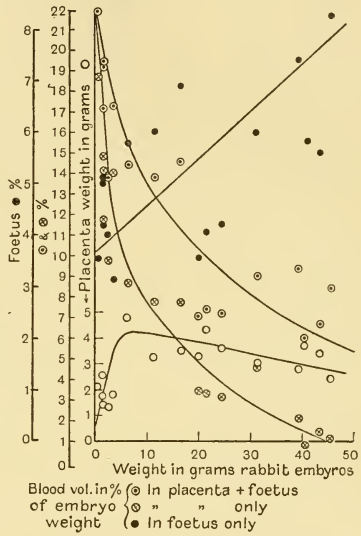


Fig. 161.

Their most important work was done on the blood-gases of the embryo. They determined the oxygen and carbon dioxide content of the umbilical artery and vein, and found what change took place during an interval of 24 minutes. From the resulting differences they did not themselves calculate the respiratory quotient, saying that a much greater number of "Doppelanalysen" would be required, but this has often since been done from their figures (e.g. by Murlin); in one of their experiments it works out at 1·6, in the other at 1·04. They calculated from their data the amount of oxygen used and carbon dioxide given out over a definite time per unit weight (metabolic rate), and obtained in the case of a sheep embryo of 1300 gm. the figure of 1·16 c.c. oxygen per kilo per minute. Comparing this with Reiset's figure for the adult sheep, of 5·8 c.c. oxygen per kilo per minute, they concluded that the embryonic metabolic

rate was four times as small as that of the adult. The same conclusions applied to carbon dioxide.

These investigations led to a long-continued discussion in which a dichotomy of opinion soon presented itself. Pflüger, who had always affirmed that the metabolic rate of embryos was far smaller than that of fully grown animals, welcomed Cohnstein & Zuntz's work as a confirmation of his views. The embryo, he said, has need for practically no muscular movement, and lives in a liquid of specific gravity very like itself, so there can be no necessity for great expenditure of energy, and therefore no "bemerkenswerthe Respiration". Gusserov took another view. Abstracting Pflüger's papers for the *Archiv f. Gynäkologie* in 1872 he said that, although it might be true that muscular motion was at a minimum in embryonic life, yet the astonishing rapidity of growth might equally well demand a considerable expenditure of energy. "You cannot overlook", he remarked, "the amazing speed with which the embryo passes from the tiniest size to the weight of the foetus at term, and this phenomenon can hardly take place without an active metabolism." Gusserov's words contain the origin of the notion of "Entwicklungsarbeit", afterwards so much elaborated by Tangl. But, although all those who took part in the controversy admitted that experiments alone could test the matter, none were carried out until 1900, when Bohr took it up anew. Cohnstein & Zuntz's second paper was only concerned with the arterial pressure before and after birth, the causes of foetal apnoea, and the first stimulus for pulmonary respiration at birth.

Bohr attacked the problem again with the advantage of improved methods, and he showed that the majority of the errors imperfectly guarded against by Cohnstein & Zuntz would act in the direction of making the metabolic rate too low. He abandoned the direct method used by them of estimating the blood-gases in the umbilical cord, and instead measured the oxygen consumption and carbon dioxide production of the maternal organism (guinea-pig) before, during, and after, clamping of the umbilical cord, i.e. cutting out altogether the influence of the embryo. In a typical experiment after compression of the umbilical cord the carbon dioxide excretion fell by 10 c.c., and the oxygen utilisation by 11 c.c., per 10 minutes. When the clamp was taken off the respiration at once rose to its former value, and fell again to just the same extent towards the end of the experiment when a ligature was put on the umbilical cord. In 10 minutes, therefore, the embryo gave out 10·5 c.c. carbon

dioxide and used up 11·5 c.c. oxygen; a respiratory quotient of 0·913. The following table gives the results obtained:

| C.c. weight of embryo | Respiration per 10 min. C.c. carbon dioxide put out | C.c. oxygen taken in | Respiratory quotient Mother | Embryo | (Carbon dioxide) metabolic rate (c.c. per kilo per hour) Embryo | Mother |
|---|---|---|---|---|---|---|
| 35·8 | 10·5 | 11·5 | 0·74 | 0·913 | 586 | 452 |
| 39·0 | 12·0 | 10·0 | 0·79 | 1·20 | 462 | 408 |
| 61·5 | 10·0 | 9·0 | 0·92 | 1·11 | 488 | 478 |
| 23·8 | 6·0 | 6·0 | 0·81 | 1·00 | 252 | 483 |
| 5·5 | 5·0 | 3·0 | 0·91 | 1·67 | 1350 | 598 |
| 16·0 | 4·0 | 4·0 | 0·87 | 1·00 | 756 | 490 |
| | | Average (excluding 5 gm. embryos) | | | 509 | 462 |

A glance at the table shows that in all cases the respiratory quotient was in the neighbourhood of unity, from which it may perhaps be concluded, though Bohr himself refrained from emphasising it, that the main source of energy in mammalian development is carbohydrate[1]. During the periods when the maternal organism alone was respiring, the respiratory quotients varied between 0·74 and 0·92, with an average of 0·84, instead of the foetal average of 1·14. Perhaps most interesting of all are the figures for metabolic rate, calculated on the basis of the carbon dioxide results. There is a fairly close correlation as regards age, for the values run in order of embryo weight, 1350 (this was regarded by Bohr as doubtful), 756, 252, 586, 462 and 488. The third of these is the only aberrant one, and a first survey would conclude that the metabolic rate declines in the guinea-pig embryo from a very early time, just as it does in the chick. This point of view, however, does not fit in with the data of the calorimetric workers mentioned below, and, as a matter of fact, Bohr himself did not adopt it. He contented himself with averaging the figures and concluding that the metabolic rate in pre-natal life in the guinea-pig was of much the same order as in the maternal organism, thus agreeing with Gusserov rather than Pflüger, and avoiding any commitment on the question of whether during development it went up or down. Bohr's position was, of course, that no true Entwicklungsarbeit was necessary, but that, at the same time, the embryonic cells were not "in a condition to exist without a vigorous metabolism". With his work all accurate information on the respiratory intensity and respiratory quotient of the mammalian embryo ceases, and there is probably no gap in our

[1] Again, Dickens & Simer found an R.Q. of 1·04 for whole rat embryos *in vitro* (see p. 703).

knowledge of the biophysics and biochemistry of the embryo at the present time so great as this.

Various investigations have been made of closely related subjects. The question of the initiation of pulmonary respiration, for instance, was thoroughly gone into by Preyer and by Bert. The passage of gases through the placenta was studied by Dubois & Regnard, by Butte and by Charpentier & Butte, who from clinical experience and a few experiments with rabbits supported Zuntz's original view that in maternal asphyxia the foetal can give up oxygen to the maternal circulation. In 1880 Hoeyghes reported that carbon monoxide would not pass the placenta, but this was shown to be incorrect by Gréhant & Quinquaud and Plottier and, later, Nicloux and Nicloux & Balthazard, examined the passage of carbon monoxide across the placental membranes in the guinea-pig. They concluded that it passed through by a diffusion process provided that sufficient time could elapse for the slow dissociation of the carboxyhaemoglobin to create an adequate pressure gradient at the placental membrane. It then appears in the blood of the embryo, but, if the carbon monoxide is given in too great amount, death of the animal occurs before these events have had time to happen, and no carbon monoxide is to be found in the foetal blood. Huggett later investigated, in some careful experiments, the question of the passage of oxygen and carbon dioxide through the goat placenta, measuring the partial pressures in the maternal and foetal bloods with a view to deciding whether diffusion or secretion held good. The results were as follows:

Table 84. *Embryonic blood-gases per cent.* (*average*).

| | Goat (Huggett) | | Sheep (Cohnstein & Zuntz) | |
|---|---|---|---|---|
| | Oxygen | Carbon dioxide | Oxygen | Carbon dioxide |
| Foetal arterial blood from umbilical vein | 7·96 | 29·9 | 6·3 | 40·5 |
| Foetal mixed blood (going to brain) ... | 5·90 | 36·6 | — | — |
| Foetal venous blood from umbilical artery | 2·94 | 41·4 | 2·3 | 47·0 |

The blood-gas tensions were as follows (in mm. of mercury):

| | Goat (Huggett) | |
|---|---|---|
| | Oxygen | Carbon dioxide |
| Foetal arterial blood from umbilical vein | 41 | 44 |
| Foetal mixed blood (going to brain) ... | 28 | 54 |
| Foetal venous blood from umbilical artery | 15 | 61 |
| Maternal arterial blood ... ... | 60 | 43 |

The difference between the oxygen tension of the blood supplied to the embryonic circulation and the blood coming away from the embryo is therefore 60 − 15, i.e. 45 mm. of oxygen—amply sufficient to allow of a diffusion process[1]. In just the same way, the difference between the carbon dioxide of the maternal arterial blood and that of the blood coming away from the embryo is 61 − 43, i.e. 18 mm. carbon dioxide, and the same argument holds. The maternal and foetal bloods could not be compared directly in Barcroft differential manometers because they have not the same dissociation curve, so Huggett used a differential tonometer method in which gas mixtures were allowed to come into equilibrium with the maternal and foetal blood, and the tensions then estimated in a Haldane gas analysis apparatus. The relative blood-gas tensions then worked out as follows:

$$\frac{\text{Maternal arterial blood (oxygen)}}{\text{Foetal venous blood (oxygen)}} = \frac{1{\cdot}9}{1{\cdot}0}$$

$$\frac{\text{Maternal arterial blood (carbon dioxide)}}{\text{Foetal venous blood (carbon dioxide)}} = \frac{1{\cdot}0}{1{\cdot}21}$$

$$\frac{\text{Maternal venous blood (oxygen)}}{\text{Foetal arterial blood (oxygen)}} = \frac{1{\cdot}0}{1{\cdot}3}$$

"The first experiment shows", says Huggett, "that the gradients existing between the foetal venous blood going to the placenta and the maternal arterial blood going to the uterus are adequate for diffusion. The third experiment shows that the maternal venous blood in the uterine vein has a lower oxygen tension than the foetal arterial blood which is not surprising if we remember that the placenta, unlike the lung, absorbs an appreciable quantity of oxygen." Huggett also did some experiments in continuation of Zuntz's original ones, in which the foetal blood gave up oxygen to the maternal blood. By asphyxia Huggett found that this can actually take place. The reversal of the gas current through the placenta when the gradient on the maternal side is reversed, though difficult to explain on any secretion theory, would be an obvious corollary of diffusion.

[1] Huggett worked with embryos at term, but Kellogg showed that the difference between $O_2$-content of maternal arterial and foetal venous blood in the dog is much greater early in development than it is later on. The difference is gradually lessened by the rising oxygen-content of the foetal venous blood. This may be due to greater oxygen-carrying capacity or to an increase in the ratio placental area/unit foetal weight. Apparently the placenta gives a low margin of safety, for the pulmonary area of the newborn infant is at least twice the placental area, and the oxygen-content of its blood twice that of the foetus at term. Nor is the blood of the latter more than 63 per cent. saturated with oxygen, although the corresponding maternal figure is 95 per cent. (Eastman).

Bell, Cunningham, Jowett, Millet & Brooks; and Haselhorst found the volume percentage of oxygen in the bloods to be as follows:

| | Bell, etc. | Haselhorst |
|---|---|---|
| Maternal arterial ... ... | 18·87 | 14·56 |
| Maternal venous ... ... | 15·44 | 10·11 |
| Foetal arterial ... ... | 9·02 | 3·53 |
| Foetal venous ... ... | 5·85 | 0·87 |

Bell also made some observations on the alkaline reserve of the two circulations, obtaining results contrary to the earlier ones of Levy-Solal, Weismann-Netter & Dalsace; Williamson; and Losee & Van Slyke. The English workers found more carbon dioxide combining power in the maternal than in the foetal blood, and the French and American workers found the opposite.

Table 85.

| | Carbon dioxide combining power in vol. % (c.c. per 100 c.c.) | | | |
|---|---|---|---|---|
| | Maternal | | Foetal | |
| Levy-Solal & associates (4 cases) ... ... | 30·4<br>48·1<br>37·8<br>42·0 | | 49·5<br>55·9<br>53·6<br>48·2 | |
| | Art. | Ven. | Art. | Ven. |
| Bell & associates (5 cases), average ... | 40·45 | 43·45 | 37·7 | 40·0 |
| Losee & Van Slyke (4 cases), average ... | 50·0 | | 53·0 | |
| Williamson (7 cases), average ... ... | 31·2 | | 34·0 | |
| Rieländer ... ... ... ... ... | — | | 37·1 | |

The rôle of the placenta in foetal respiration has also been studied by Schmidtt, who in a series of papers has put forward the suggestion that the foetal respiratory centre is situated, for a time at least, in the placenta, and not in the embryonic medulla. By perfusing the placenta with various solutions, and by studying the effect of altering the *p*H of the perfusing fluid, he found that the acid side would cause vaso-dilation and the alkaline side vaso-constriction, so that a regulatory mechanism of some sort was evidently present[1].

## 4·16. Heat-production of Mammalian Embryos

We may now return to the heat-production of the mammalian foetus. Unfortunately this has never been measured directly, for the technical difficulties in doing so have so far been insuperable, and all that we know about it is derived from experiments in which the

[1] There are, of course, no nerves in the mammalian placenta (Ikeda).

maternal organism enters in as a disturbing factor. Much attention has been given to the basal metabolism of women in pregnancy, and, although the conclusions about the embryo are not too certain, it is necessary to review them carefully.

In 1908 Rubner expressed the belief that his surface area law applied not only to the newly born animal but also to the embryo. As the average weight of an individual human infant at birth is 8 per cent. that of the mother, Rubner calculated that the metabolic rate of the foetus at term would be approximately twice the maternal metabolic rate, but, because the foetus is not very active, its rate would be less than that. Such a point of view agreed well enough with the experimental values of Bohr. But it was very difficult indeed to know what part the new tissues would play in the total heat-production of the mother plus the foetus as a unit, for, although the embryo itself might have a much higher metabolic rate than the mother, the fluids, the umbilical cord, the membranes, etc., would have a very low one or none at all, while that of the placenta was more or less incalculable. The earlier observations on pregnant animals, moreover, gave conflicting results. Reprev could find no increase in basal metabolic rate in the pregnancy of the rabbit, guinea-pig, and dog, while, on the other hand, the figures of Oddi & Vicarelli on mice showed a marked increase. This increase was also found by Magnus-Levy, who carried out the first reliable observations on a pregnant woman; in this case the rate rose from 2·8 c.c. oxygen per kilo per minute in the 3rd month to 3·3 c.c. in the 9th month (17 per cent.). L. Zuntz however, did not find such a rise. Murlin in 1910 was able to show that the extra heat-production during pregnancy in the dog was almost exactly proportional to the number of embryos, i.e. the total weight of the litter. The experiment was done on two pregnancies of the same dog. The figures were as follows:

| | Gram calories produced per day |
|---|---|
| During sexual rest (normal) ... ... | 505·3 |
| During first pregnancy ... ... | 551·3 |
| During second pregnancy ... ... | 763·8 |

In the first pregnancy 1 puppy was born, in the second 5. $551{\cdot}3 - 505{\cdot}3$ gave 46·0 cal. per puppy, or, as it weighed 280 gm., 16·4 gm. cal. per 100 gm. Similarly $763{\cdot}8 - 505{\cdot}3$ gave 258·5 cal., or 51·7 cal. per puppy, or, as they weighed 312 gm. each, 16·8 gm. cal. per 100 gm. If now it could be shown that the metabolic rate of the maternal organism

remained perfectly constant during pregnancy, there was a possibility of determining how the foetal metabolic rate varied. Murlin also estimated the heat-production throughout pregnancy, and a curve plotted from his figures is shown in Fig. 162. No appreciable increase occurred until gestation had been half accomplished. Murlin calculated that the extra metabolism due to the embryo (and all accessory structures) at the end of pregnancy was almost exactly equivalent to the amount which a newly born animal of the same weight would theoretically produce (according to Rubner's skin-area law) if exposed to ordinary room temperature and resting. Thus in the case of the 1-puppy pregnancy, the extra metabolism was 46·0 cal., and the extra metabolism calculated by Meeh's formula from the embryo-weight was 45·4. Similarly the extra metabolism in the case of the 5-puppy pregnancy was 258·5, and the same calculated from the embryo-weights was 251·6 gm. cal. Thus the total curve for mother and offspring should not suffer any change at birth, if all muscular movement were abolished. Murlin & Carpenter were later able to verify this in the case of man where there was no muscular movement.

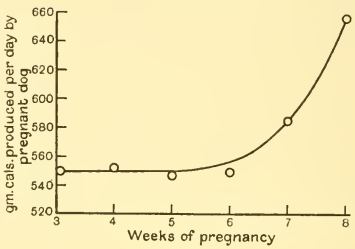

Fig. 162.

The estimations of L. Zuntz; Murlin & Carpenter and Hasselbalch all agreed in showing an extra basal metabolism near term of about 4 per cent. Apart from this small increase the heat given off per unit weight per unit time, according to Murlin, was the same as under normal conditions, i.e. the embryo functions as so much maternal tissue, its higher metabolic rate being just counterbalanced by the inactive and relatively inactive structures. Another exact compensation was that the increase in oxidation of the infant's body when it passes from the warm environment of the uterus to the cold of the outside world was almost exactly equivalent to the oxidation rate of the accessory structures that supported it *in utero*.

Other researches on the basal metabolism during pregnancy in man are those of Baer; Cornell; Wilson & Bourne; Haselhorst & Plant; Root & Root; Sandiford & Wheeler and Rowe, Alcott & Mortimer. The first two of these sets of data are believed by Harding to be faulty,

but the others equate well with those of Magnus-Levy, and form the basis of our knowledge of the process. The gradual rise of heat-production during pregnancy is particularly well shown in the figure of Root & Root, reproduced here as Fig. 163. From the 6th month it steadily rises until, at 6 weeks before birth, it is 23 per cent. higher than at 4 months. The total increase in weight, however, was only 14 per cent., and a non-pregnant woman showing a similar increase

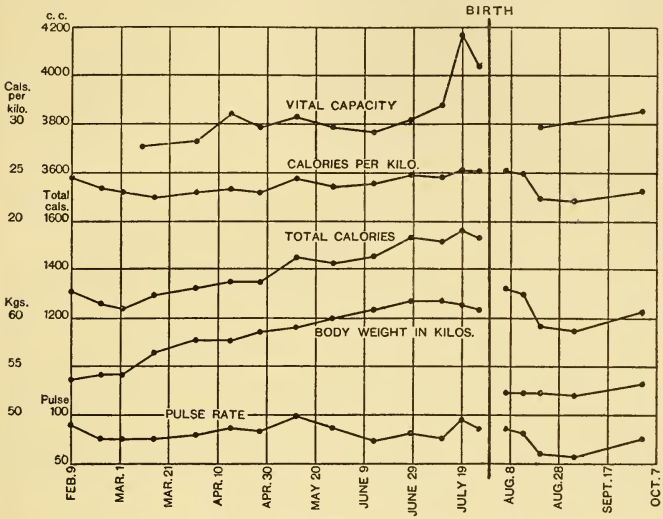


Fig. 163.

in weight would only have increased her heat-production by 5 per cent., according to the tables of Harris & Benedict or Aub & Dubois.

Similar results were obtained by Sandiford & Wheeler. Murlin & Carpenter had shown in 1911 that if the energy exchange of a pregnant woman at the 9th month were compared with the energy exchange of the mother *post partum*, the metabolism total in each case was exactly the same except for a balance of 4 per cent. in favour of the pregnant woman. This means that there is no deflection in the energy consumption curve at birth, that the maternal organism and the foetus function as two separate units in their consumption of energy, and that the rise of heat production during

pregnancy is entirely due to the embryo. This attitude is adopted by Garipuy & Sendrail as the result of their work[1]. Sandiford & Wheeler have made this point of view very much the most satisfactory by calculating from their own figures and those of all other observers the (basal) metabolic rate of the embryo on this assumption. The weight of the foetus was obtained by means of the standard curves for human pre-natal growth, and Lissauer's formula for infants ($10{\cdot}3 \times \sqrt[3]{W^2}$ instead of Meeh's $12{\cdot}3 \times \sqrt[3]{W^2}$) was used to calculate its surface at the different stages. Then the weight and surface (Dubois charts) of the pregnant woman being known, and the weight of the foetus subtracted from it, the metabolic rate of the mother alone could be calculated. The result was that the sum of the two agreed remarkably well with the figures actually found experimentally.

More explicitly what Sandiford & Wheeler did was this. Sandiford had previously calculated the surface area of the human embryo at different stages, and this value, added to the calculated surface area of a woman equal in weight to the pregnant woman minus the foetus, gave the total surface in question. Then, when the total calories eliminated by mother and foetus were divided by the sum of the surface areas so obtained, the resulting figures would represent the heat production of a unit mass of active protoplasmic tissue. It was found that actually there was no significant change during pregnancy in this value, which remained constant within small limits of variation at 35 calories per square metre per hour. Sandiford and Wheeler pointed out that this calculation would be invalid if surface area law depended on Newton's Law of Cooling, but was valid if it depended, as in their opinion and in that of Boothby & Sandiford, it did, on a proportionality between surface area and mass of active protoplasmic tissue. I shall again return to this point. They also used another method of calculation. They assumed that the heat-production per unit mass of foetal tissue was constant throughout foetal life, and, by multiplying the heat-production for each square metre of body-surface each hour by the surface area of the foetus corresponding to its estimated weight, the total calories each hour for the foetus were obtained for the various months. If this latter figure was subtracted from the total calories of mother and foetus, the total calories of the mother alone were obtained,

[1] See also Pommrenke, Haney & Meek.

and when divided by the estimated surface area of the mother, the calories per square metre of body-surface for mother alone were practically the same as those found by the other method. But they were not quite the same, and the difference between them was always greater towards the end of pregnancy than at the beginning—a fact for which Sandiford & Wheeler offered no explanation, but which may be due to the fact that the heat-production per unit time per unit mass of foetal tissue is probably not the same throughout development. The general trend of all that has already been said works strongly against the assumption that it is. The various curves which have been mentioned are shown in Fig. 164 taken from Sandiford & Wheeler's paper. Curve *A* represents the calories for each kilo (in this instance not rising very much), curve *B* the basal metabolic rate calculated during the course of pregnancy by dividing the total calories put out each hour by the sum of the surface areas of mother and foetus, and comparing the result obtained with the Dubois normal of the mother, 36·5 calories. Curve *B′* shows the basal metabolic rate calculated in the usual way, using the Dubois surface area and the normal standards. Curve *C* shows the calories per square metre per hour calculated in the same way as for curve *B*, and curve *C′* shows the calories per square metre per hour obtained by dividing the total calories each hour by the Dubois surface area obtained by using the total weight of mother and foetus in the usual manner. Curve *D* represents the total calories per hour eliminated, i.e. the raw data of heat given up to the calorimeter.

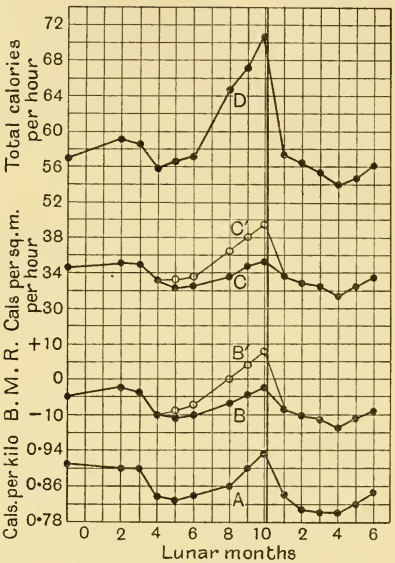


Fig. 164.

The general impression left by the work of Sandiford & Wheeler and of the other investigators mentioned is that the metabolic rate of the human embryo does not change much during pre-natal life.

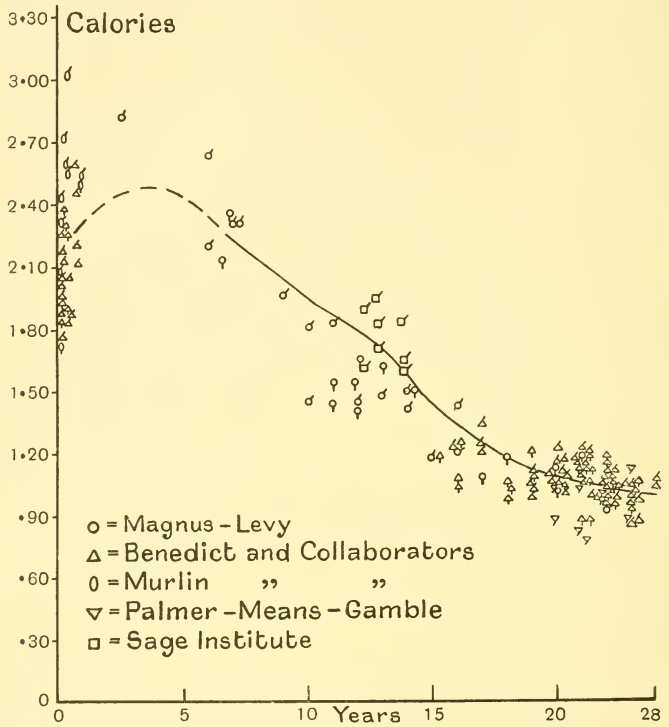


Fig. 165.

But it must be remembered that they could not take account of the embryo until the 4th month, owing to its minute weight compared to that of the mother. On the whole, however, the measurements of heat-production during pregnancy are very difficult to interpret from the point of view of the present discussion.

After birth, the metabolic rate is not a constant, nor does it begin to decline immediately. In man it rises until about the 4th year, after which it declines continuously except for a slight kink at 12·5 years, believed to be associated with puberty. The classical paper in which this peak was observed is that of Dubois, which appeared in 1916, but exactly the same state of affairs is indicated in the papers of Benedict & Talbot; Murlin & Hoobler; Murlin & Bailey; and Marine, Lowe & Cipra. Dubois himself made many measurements of the metabolic rate of children from 6 to 14 years of age, and compared them with those of many other observers for the periods before and after these limits. These papers should be consulted for the relevant literature. In Fig. 165, taken from his paper, the resulting curve is shown, and, as has already been said a definite peak at 4 years of age is to be seen. This curve is in every way comparable with that in Fig. 133, where the data of Gayda on the metabolic rate of the toad are plotted. The figures of Magnus-Levy & Falk, moreover, show the same peak on the basis of carbon dioxide and oxygen determinations. Clearly if this peak were real, it would be expected that infants born some time before term would show a basal metabolism below that of normal infants. This was actually found to be the case by Marsh; Murlin & Marsh; Talbot & Sisson and by Talbot, Sisson, Moriarty and Dalyrymple, whose figures agree very well and demonstrate a little further the earliest part of the Dubois curve.

Just as in the case of the toad the peak occurs not very long in the life-span after hatching, so in the case of the human being it occurs not very long after birth. But these two organisms are not the only ones for which a peak of metabolic rate has been demonstrated, for Deighton & Wood in 1926, using Capstick's calorimeter, found an exactly similar one in the case of the pig. Fig. 166*a* shows the results obtained. From birth the pig's metabolic rate rises rapidly, reaching a maximum of 72 calories per square metre per hour at an age of 4 months, after which time it falls away less rapidly than it rose, and this peak holds true also when the heat-production is plotted against the weight. Not only the nature of the curve but also even its general form are in close agreement with the work of Dubois and many others on man, and Gayda on the toad. But Wood and his assistants found that not all breeds of pig gave a peaked curve. If the Berkshire breed was used instead of the Large White, the falling part of the curve appeared but not the ascending part, as is

indicated on Fig. 166 *a* by the small crosses, so that in this other breed the peak, if there was one, occurred before birth. That there had been one Wood had no doubt, for if the curve had been simply continued in an upward direction with decreasing age, the embryo of a few grams would have been radiating heat to the intensity of a red-hot body. The descending metabolic rate curves must, indeed, in all cases, come from a peak, and not from some indefinitely high level.

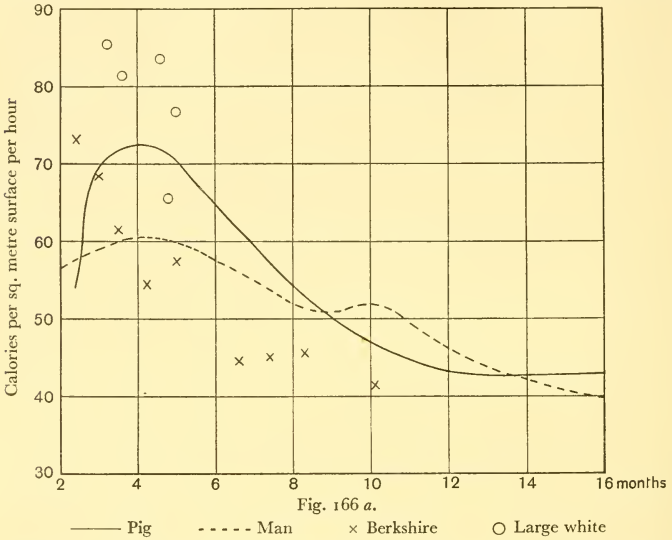


Fig. 166 *a*.

Subsequent work by Deighton on other breeds of pigs has carried further the work reported in Wood's paper, and has demonstrated that in some cases the peak occurs after birth, in other cases, before it. Russell, who hoped to find an extra-uterine peak in the rabbit, which is born relatively early, and an intra-uterine peak in the guinea-pig, which is born relatively late, found a curve for the former animal which resembled that of the Berkshire pig. Ginglinger & Kayser, however, did succeed in finding such a post-natal peak in the case of the rabbit, as may be seen from Fig. 166 *b*. They contrast the curves given by the pigeon (showing neither chemical nor physical heat-regulation at birth) with the rabbit (which shows chemical regu-

lation only) and with the guinea-pig (which can fully control its temperature at birth). Correspondingly the guinea-pig—like the Berkshire pig—obeys the classical rule of descending heat-production from birth onwards; these animals have, as it were, settled on their thermal neutrality point before birth and as it is kept steady we can observe the effects of decreasing relative surface. The pigeon and the mouse, on the other hand, are born without any regulatory power, and their metabolic rate goes on increasing until this is attained. Intermediate between these two groups come such animals as the rabbit, which can

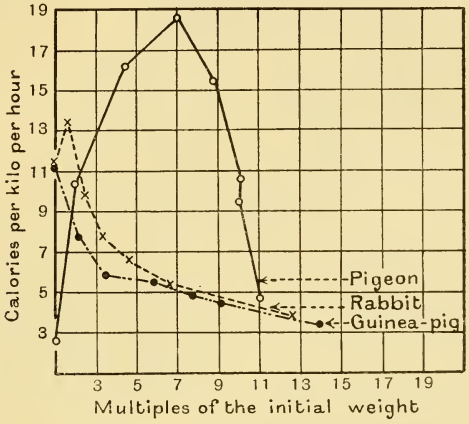


Fig. 166 *b*.

resist cold at birth but not heat, i.e. which have a partial regulatory power. These show an increasing metabolic rate for a short time only, and the curve for the rabbit in Ginglinger & Kayser's figure (166 *b*) compares interestingly with that for man in the graph of Dubois (165)[1]. And the fact that the chick's metabolic rate is declining throughout its incubation-period from the 5th day onwards would agree with the finding that its heat-regulative power is fully developed at birth. The peak of metabolic rate in mammals is explained by Ginglinger & Kayser, then, as being due to their varying degrees of heat-regulative power, but would this explain the peak on Gayda's curve for the toad, which never succeeds in regulating its temperature at all?

[1] And with those for growing calves given by Brody.

## 4·17. Anaerobiosis in Embryonic Life

Very few observations have been made of the respiration of embryo cells in tissue culture, but Burrows has some interesting experiments in this direction. Taking explants of chick embryonic heart cells, he placed them in different partial pressures of oxygen, and found that their behaviour was quite different according to the stage of development of the chick from which they had been taken. The results he obtained are pictured in Fig. 167, from which it can be seen that fibroblasts from 4–5-day embryos would grow and pulsate for 46 hours or so in pure nitrogen, and for 50 hours in only 7·5 per cent. of oxygen. Fibroblasts from 10–15-day embryos, however, would not grow at all in nitrogen, and only for 12 hours in 1·5 per cent. oxygen. Burrows was in doubt as to the meaning of these phenomena, but explained them by the hypothesis that there must be some source of energy contained in the young cells, which the older ones have not got. One is reminded, on the one hand, of the work of Cohn & Murray on the growth-rate of embryo explants, and, on the other hand, of the concept of "ontogenetic momentum" which Byerly's results on asphyxiated embryos (see p. 607) and de Bruyne's results on embryo autolysis (see Section 14·11) have brought into being. Consideration of it will be postponed till later in the book. Burrows found, in a word, that with age the property of being able to grow anaerobically declined, and was eventually lost. This important result was fully confirmed by Wind, who modified it by using really strict anaerobic conditions. Under these only the very slightest amount of growth went on even with heart-cells from 4–5-day old embryos, but when an atmosphere of $2 \cdot 10^{-4}$ vol. per cent.

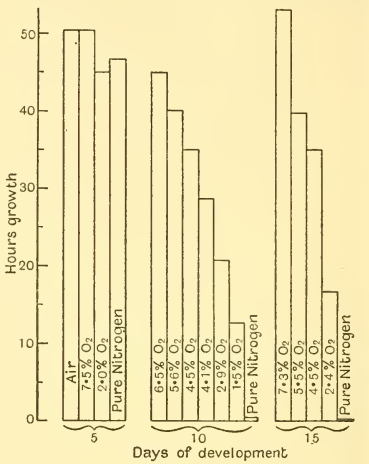


Fig. 167.

of oxygen was used, the difference was unmistakable, for cells from 10-day old embryos would not grow at all, while those from 5-day old embryos grew excellently. Wind also made the very interesting observation that, if the cells were in each case sub-cultured for many weeks and then brought under anaerobic conditions, the difference between the cells from 10-day embryos and those from 5-day embryos had entirely disappeared. No difference in growth was now perceptible.

Work along these lines was continued by Wright whose results may be summarised in the following table:

Table 86.

| | | Mm. mercury pressure of oxygen |
|---|---|---|
| Wright | Explants of 10th day chick embryo myoblasts: | |
| | (*a*) In 0·22 % glucose: mitosis ceases at ... | 18·6 |
| | emigration ceases at ... | 12·4 |
| | (*b*) In 0·06 % glucose: mitosis ceases at ... | 14·6 |
| | emigration ceases at ... | 9·0 |
| ,, | Jensen rat sarcoma: mitosis ceases at ... ... | 6·0 |
| ,, | Mouse carcinoma: mitosis ceases at ... ... | 3·0 |
| Ephrussi, Chevillard, Mayer & Plantefol | Explants of 8th day chick embryo heart fibroblasts: mitosis (but not movement) ceases at ... ... | 7·0 |

These facts lead naturally to the consideration of the evidence which has been brought forward from time to time in favour of the view that during the early stages of embryonic development anaerobiosis may be possible, or may even normally occur. There is no need to dwell on the first efforts of the workers on the hen's egg, for their experiments have already been briefly described. Nor can cleavage of echinoderm eggs go on in the absence of oxygen. Some recent careful experiments of Amberson on the eggs of *Arbacia punctulata* have demonstrated that cleavage proceeds at normal rate down to very low oxygen tensions—about 11 mm. of mercury, between which point and 4 mm. the rate is slowed down, while below 4 mm. cell-division in this egg will not go on at all. Drastich finds exactly parallel effects in the case of *Strongylocentrotus lividus*. In the case of the nematodes, again, Zavadovski showed in 1916 that cleavage in *Ascaris megalocephala* would not go on in the absence of oxygen and that the reason why it stopped in putrefying media was because the bacteria were successfully operating a prior claim for the oxygen present. More recently, Zavadovski & Orlov have demonstrated the absolute dependence of many nematode embryos on oxygen for their cleavage,

and Kozmina, working on *Ascaris*, has obtained very similar results to those of Drastich, mentioned above. On the other hand, the formation of the chitinous envelope, the internal membrane and the perivitelline space, and the elimination of the two polar bodies, can go on normally in the absence of oxygen, as appears from the work of Szwejkovska. As regards cleavage, Szwejkovska is in agreement with Kozmina and with Zavadovski & Orlov.

There is little evidence that developmental rate in any form is increased by raising the oxygen tension or concentration. Rollat's claim that silkworm eggs hatched much earlier in compressed than in ordinary air was discredited by Bellati & Quajat.

The disputed question only concerns amphibia and birds, and originates from the work which Samassa did during the last decade of the last century. We are not here concerned with variations in the degree of susceptibility to oxygen lack or oxygen excess on the part of the embryo during its development, but with the capacity which it has been alleged to have of being able to live and develop anaerobically in the very early stages. Samassa affirmed in his first paper that the early segmentation stages of frog's eggs were apparently independent of oxygen, and would proceed in atmospheres of hydrogen and nitrogen, though gastrulation would not take place under such conditions. Kept in pure irrespirable gases, they retained, if unfertilised, their developmental capacity for many days, though the resulting embryos were often abnormal. The experiments were done in a stream of pure hydrogen, so that Samassa believed he had washed every trace of oxygen out of the gelatinous egg-coverings. He next tried high vacua, first by means of a mercury pump, and then a cathode ray. Still they developed as far as complete blastulae. Samassa found, however, that carbon dioxide had a definitely toxic action, stopping segmentation, and, if pure, killing the eggs within 20 hours. (Compare Burfield's work on the plaice egg, p. 669.) He recalled that Hallez had found *Ascaris* eggs to be capable of living for a month in pure carbon dioxide and then developing, but rightly regarded a parasitic nematode as a special case. About the same time Loeb reported an extreme resistance on the part of *Fundulus* eggs to oxygen lack in the very early stages. Samassa was convinced that the effects he obtained were not due to traces of oxygen, and argued that, if it were so, all the eggs in a vacuum flask would hardly be expected to develop synchronously, for the weakest would be

weeded out, yet this was never the case. Again, a high population of eggs would be very injurious, yet this was not so. Then it might be supposed, if traces of oxygen were responsible, that admission of air and re-evacuation would lead to further development, but this was not the case. In atmospheres of 20 per cent. oxygen and 80 per cent. hydrogen, the eggs developed just as normally as in the pure hydrogen. Loeb supported Samassa's conclusions, working with *Ctenolabrus* and *Arbacia* eggs. In atmospheres of hydrogen, he found, development would stop, but by no means immediately; thus the eggs of *Fundulus* (a bottom fish) could last 15 hours in complete absence of oxygen, segmenting, and retaining perfect viability even after 4 days' anaerobiosis. The eggs of *Ctenolabrus*, on the other hand, were very sensitive to carbon dioxide, and a stay of only 4 hours in that gas killed them altogether. *Arbacia* and *Paracentrotus* eggs were held up at once in the absence of oxygen, a fact subsequently confirmed by Warburg. Again, in later stages, the hearts of *Ctenolabrus* and *Fundulus* behaved rather differently. In 10 mm. partial pressure of oxygen the heart-beat of *Ctenolabrus* embryos was quite abolished, but that of *Fundulus* embryos could proceed, if slowly, for as long as 9 hours, and then completely recover. Carbon dioxide was equally toxic for both hearts, and hydrogen would act as an antidote to its action so that the heart might begin beating again in hydrogen when that gas was substituted for carbon dioxide.

O. Schultze opposed these conclusions. He maintained that the technique of the other workers had been faulty, and that traces of oxygen had been present. As a means of removing all such traces from the air, he adopted the ingenious expedient of using the eggs themselves. The eggs were placed in a tube (of just the right size to fit them) passing through a cork. After 2 days, eggs Nos. 1 and 8 (those nearest the open ends of the tube) were gastrulating, 2 and 7 were beginning to do so, while Nos. 3, 4, 5 and 6 had all stopped in the earliest cleavage stages. When all were turned out into a dish, however, all developed normally. Schultze concluded that small amounts of air must have been present in the other experiments, but it is as a matter of fact very difficult to see from Samassa's account of them how this can have been so. As for Loeb's results, Schultze interpreted them as being simply due to a difference in oxygen requirement as between the two embryos. Nevertheless, investigators continued to report confirmations of Samassa's experiments, notably Godlevski in 1901, and contra-

dictions of them, such as Wesselkin in 1913, who worked with chick embryos, and found that an atmosphere of 5 per cent. oxygen killed them in 48 hours, but that 10–15 per cent. permitted continued development for 72 hours. It may be concluded that, although there is no strong reason for believing that embryonic development can ever go on anaerobically, there are yet some curious facts which ought to be looked into before a final decision is reached. And the work of Reiss & Vellinger referred to on p. 869 suggests that the energy required for cleavage may be obtainable without free oxygen, by electron transfer.

## 4·18. Metabolic Rate in Embryonic Life

Attention must now be directed for the last time to the metabolic rate question. We have already seen that Gayda (for the toad), Wood (for the pig), Dubois (for the human being) and Ginglinger & Kayser (for the pigeon and the rabbit) have shown that the respiratory rate has a point of maximum intensity during the life-span, though subsidiary kinks on the curve may exist (as at puberty). It is extremely probable that, in the rising rate curves of echinoderms and amphibia early in development, we see the ascending part of a curve, and, in the falling rates of avian embryos, we see the descending part of the same curve. What factors determine the point in the life of the individual at which the peak shall occur are as yet obscure although, as we have seen, Ginglinger & Kayser explain it by the onset of heat-regulation.

It must first be pointed out that the falling part of the curve has often been plotted, and is well seen in the measurements of Magnus-Levy and Falk on man; of L. Mayer on the hen, the duck, and the guinea-pig; of Sayle on dragonfly larvae; of Krarup on rabbits and of Benedict & Riddle on pigeons[1]. Mayer noticed that, in the case of the guinea-pig, the fall after birth followed the course of a regular hyperbola. The following figures, again, exemplify it.

| | C.c. carbon dioxide put out per kilo per hour |
|---|---|
| Very small rabbit embryo (Bohr) | 750 |
| Rabbit embryo nearly at term (Bohr) | 509 |
| Adult rabbit (Krarup) ... ... | 450 |

Its extremely general character is shown by the fact that Hée obtained a curve for intensity of gaseous exchange during the develop-

[1] Other instances are cows (Brody); cladocerans (Obreshkove); molluscs (Hopkins).

ment of the mould *Sterigmatocystis nigra* which closely resembled that of a growing population of cells such as the chick embryo. Among the most important of the suggestions that have been made with regard to it is the notion of the "masse protoplasmatique active", in contrast to the "ballast", which was introduced by Friedenthal, and has since been much developed by the Strasburg school (Faculty of Medicine). As differentiation goes on, and the make-up of the embryo becomes ever more and more complicated, there must be a constant increase in the amount of storage substances which have themselves no respiratory function, but which participate in the total weight of the embryo. These substances, which are known as "substances paraplasmatiques", will, if they increase out of proportion to the size of the growing embryo, obviously have the effect of lowering its unit respiratory activity. Cohn & Murray suggest that the lipoid granules of the central nervous system and the brown pigment of the heart and liver may be substances of this class. Kassowitz and Mühlmann have discussed the accumulation of "metaplastic" bodies in cells during the growing process.

Another and most important factor which has to be taken into consideration in this connection is the relation between the surface and the volume. As Cohn & Murray put it, "It is in the very nature of geometric relations that with growth the volume or mass increases as the cube, and the surface as the square of a number. The result from a biological standpoint is that for a unit mass of active protoplasm undergoing continuous chemical changes, the portals, that is to say, the surfaces of the organism for entry and exit of the substances which are the antecedents or products of vital activity become continuously smaller, and therefore continuously less suitable for maintaining the original velocity of metabolism. There must necessarily follow a diminution of activity and all the other changes that are merely the logical outcome of the initial modification."

The surface/volume theory must, however, be used with care, for it has various implications other than those which appear at first sight. If the embryo maintained a perfectly spherical shape as it grew, then the theory could be applied to it in its simplest form, but the active surfaces are so numerous and so large that a great number of complicating factors must, at any rate, be admitted into the discussion. Thus in the case of the chick embryo, its effective surface is not only its skin, but also a collection of structures such as the

membranes of the allantoic and amniotic sacs, the blastoderm covering the vitelline membrane, and the renal tubules and glomeruli. As the chick is a metazoan animal, there are also the individual surfaces of the cells to be considered. Murray has summarised as follows a number of the points which have to be borne in mind in considering this question. "(1) In the case of individual cells of metazoa which are, as far as we know, of about the same size throughout life, the average of their surface/volume ratios would not change with development any more than it would in a growing colony of unicellular organisms. (2) The surface/volume ratio may theoretically be maintained at any level simply by the infolding or wrinkling of the surface, as is seen in the intestines. (3) In actuality the area of capillary surface is adjustable since the development of new vessels such as is seen in the processes of repair may occur as the result of the repeated vigorous functioning of a part. (4) It is known that under normal conditions only a fraction of the capillaries and therefore of an exposed surface is open or active at any one time. For instance the amount of heat-radiation from the skin actually depends less upon the measured skin surface than upon vascular changes, which, in turn, depend upon the metabolic rate rather than *vice versâ*. (5) It is not only the area of the surface but the permeability of a surface that is important, and as the chemical constitution of each cell changes markedly with age, so will the surface permeability change. (6) The hypothesis that growth is correlated with the area of absorptive surface supposes that through a given unit of surface a certain restricted number of molecules may pass in unit time. But if, as we know, there is change with age in the kind and therefore the size and migration-rate of molecules which enter the cell, one would hardly expect this simple relationship to be maintained. (7) Growth or storage is the difference between absorption and elimination. Either one of these factors may vary more or less independently of the other and thus growth is necessarily dependent on both of them. As the ratio of storage to elimination changes with age, if absorption is dependent upon surface, growth cannot be, and *vice versâ*." It is nevertheless much to be wished that accurate measurements were available of the extent of the active surfaces in the chick embryo at all stages of its development.

The whole question is, of course, bound up with the controversy on the relation between heat-production and heat dispersal, "thermo-

génèse" and "thermolyse", a controversy now almost a century old and still vigorously proceeding. The two points on which all the disputants are agreed are (1) that animals give off to the calorimeter more calories per kilogram the smaller they are, and (2) that, per square metre of skin surface, they all give off much the same amount of heat. These two generalisations apply certainly to homoiotherms, and probably also to poikilotherms, with certain reservations. But the great divergence of opinion arises when we try to decide whether the surface area is the cause or the effect of the heat loss. Two schools of thought have come into being on the question. For one school the essence of the interpretation is Newton's law of cooling; a given amount of surface necessitates inexorably a certain loss of heat, which must consequently be supplied by the protoplasm of the body; "thermolyse" is the cause of "thermogénèse". The factors which cause this intenser metabolism in the case of the smaller creatures, i.e. those which have most surface in proportion to their weight, are, for the adherents of this view, all of one kind, and involve differences only of degree. These differences are regarded as being due to anatomical factors. "The tissues of the various homoiotherms—very similar in composition", says Terroine, "have a more or a less intense metabolism because by the perfectly coherent operation of the circulatory and respiratory apparatus, they receive in unit time variable amounts of food and of oxygen." Obviously on this view all protoplasms are identical, and the protoplasm of the egg of a mouse could equally well go to form an elephant if it were not for the fact that the eventual form, shape, size and anatomical arrangement of the mouse exists *in potentia* in the egg-cell of the mouse, ensuring that the end-product of development shall be an object like a mouse with a proportionately large surface. The mouse protoplasm is, as it were, exactly the same as the elephant protoplasm, but destined to work a great deal harder because something in the mouse egg arranges that development shall stop when a certain small size is reached, and therefore that the energy turnover in unit time shall be considerable. The eventual surface and the eventual morphology, on this view, are what is originally given in the egg-cell—an almost Aristotelian conception which suspiciously resembles the proposition "au commencement était la forme". Perhaps we may see in this attitude another expression of that point of view which has been so ably discussed by E. S. Russell in his book *Form and Function*.

Those who have not accepted these opinions have chosen rather to agree with St Paul's affirmation that "all flesh is not the same flesh, but there is one kind of flesh of men, another flesh of beasts, another of fishes, another of birds", and to maintain that protoplasms are not identical. A mouse differs from an elephant not because the surface was the element given in the first instance, and heat must be provided to compensate for that escaping at the surface, but because the metabolic constitution of its protoplasm differs, and therefore its surface. Or, in other words, the circulatory and respiratory systems do not govern the respiratory intensity of the tissues, but on the contrary were themselves laid out to meet a certain demand. "Thermogénèse", in other words, is for these thinkers the cause of "thermolyse". An animal grows until it reaches a point at which its surface cannot be further reduced proportionately to its weight without involving a failure to carry away the appropriate portion of heat generated. An animal has, or may have, a surface proportional to its heat-production, and not a heat-production proportional to its surface. The fact that the heat loss per unit surface is much the same in most animals is regarded as a coincidence due to a curiously exact concordance between surface and active protoplasmic mass, due perhaps to the fact that both of them have a regular relation with the weight in normal cases. As for the egg, it does not contain the potential surface of the animal, save indirectly, for it consists of protoplasm capable of a definite metabolic intensity, or rather of following a definite curve till it arrives at a definite metabolic intensity, and upon that eventual intensity the eventual surface of the organism will depend.

The names associated with the first of these two points of view are numerous. Von Bergmann, one of the earliest workers on basal metabolism, advocated it, and in the earlier papers of Rubner it was fully adopted. In France, Richet consistently made it the basis of his opinions on these problems, and recently it has found a very vigorous defender in Terroine. The second of the two points of view was that originally held by Sarrus & Rameaux in 1838, and subsequently by von Hoesslin; Krogh; Pütter; and Pfaundler. Its principal representatives recently have been the American school (Benedict; Lusk; and Boothby & Sandiford), and certain continental workers, such as Noyons; LeBreton; Schaeffer; and Kayser. It is certainly not possible yet to decide which of the two great groups of investigators

is in the right. But the well-known experiment of placing a homoiothermic animal at thermal neutrality so that there is no inducement for it to give out heat, with the result that it still does (Rubner and Terroine & Trautman), is not favourable to the Newton cooling law. Benedict again, working with abnormal weight/surface relations in man, such as occur in athletes or very obese men, in atrophic children or in men without limbs, found greater variations from the surface law than could be accounted for as experimental error. Benedict; Pfaundler; and LeBreton were all led to speak of an active protoplasmic mass, with which the actual surface might or might not be in exact direct proportional relation. Terroine, while accepting the notion of active protoplasmic mass to a certain extent, held that it was just that factor which had been adapted to the surface, and so remained firm in his conviction that the surface was all along the dominant factor. One fact, indeed, seems to have remained quite unmentioned in the various discussions which have taken place on these subjects, namely, the rising metabolic rate of echinoderm, molluscan and amphibian embryos. There the surface is moment by moment getting smaller and smaller relatively to the increasing weight of respiring protoplasm, and yet the metabolic rate, whether expressed as gram calories produced per gram per hour, or oxygen taken in or carbon dioxide eliminated per gram per hour, is steadily rising. If the surface were always the responsible factor this could not be taking place. It was indeed always a little difficult to understand what the adherents of the first of the two views (namely that "thermolyse" is the cause of "thermogénèse") imagined to take place during embryonic development, for in the early stages the surface would be far greater in proportion to the weight than at any other time during life, and the egg-cell would be hard put to it to satisfy the heat-dispersing demands of its surface. Again, as Deighton and many others have pointed out, if the metabolic rate of the higher animals fell during embryonic development at the same velocity as afterwards during post-natal life, the single egg-cell must have been practically red-hot. That a peak on the curve must exist was overwhelmingly likely *a priori*, and, as regards the toad, the pig, and man, it has been actually found, but the existence of such a peak can hardly be allowed for on the von Bergmann-Rubner-Richet-Terroine theory, for, as far as we know, it is not associated with any considerable changes of surface area, and on their views it would have to be. It

must be admitted that the probabilities are much in favour of LeBreton's interpretation, as far as the embryological evidence is concerned. In post-natal life, as we have seen, it has often been assumed that the surface is somehow proportional to the active mass, but in the embryo this may not be so. The individual organism, as regards its protoplasmic metabolic intensity, follows the curve of its species, and comes at last to an equilibrium point, at which just enough surface has been developed to carry away conveniently the heat arising from its hereditarily determined protoplasmic metabolic intensity.

The surface is not the only entity that grows more slowly than the total body-weight. Brody showed that the instantaneous percentage growth-rate of oxygen utilisation and carbon dioxide elimination in the chick embryo is not the same as that of the whole body[1]. This affords an obvious indication of an active protoplasmic mass growing (like the surface) less rapidly than the embryonic body as a whole. If Brody's instantaneous growth-constants are compared they work out as follows:

Table 87.

| | Time taken to double the entity | | | Instantaneous % growth-rates | | |
|---|---|---|---|---|---|---|
| Carbon dioxide production | | | | | | |
| Days | 0–4 | 4–14 | — | 0–4 | 4–14 | — |
| Atwood & Weakley ... | 0·7 | 2·2 | — | 98 | 31 | — |
| Murray ... ... ... | — | 1·9 | — | — | 36 | — |
| Hasselbalch ... ... | — | 2·2 | — | — | 32 | — |
| Growth in wet weight | | | | | | |
| Days | 4–8 | 8–12 | 12–16 | 4–8 | 8–12 | 12–16 |
| Lamson & Edmond ... | 1·2 | 1·9 | 2·9 | 56 | 36 | 24 |
| Days | 6–10 | 10–14 | 14–18 | 6–10 | 10–14 | 14–18 |
| Hasselbalch ... ... | 1·2 | 2·4 | 3·6 | 56 | 29 | 19 |
| Murray ... ... ... | 1·5 | 2·1 | 3·1 | 47 | 33 | 21 |

"Either the $CO_2$-producing mechanism", says Brody, "develops at a constant percentage rate independent of the increase in body-weight, or the weight of the body or its constituents cannot be taken as an index of the growth of metabolising tissues." These important facts illustrate from an unusual angle the constancy of heat output per unit surface. The surface is not the governing factor, but rather something else growing also more slowly than the total body-weight.

There is no need to discuss the attempts which have been made to identify chemically this active protoplasmic mass, or the paraplasmatic proteins, for they do not directly concern the embryo.

[1] See Figures 52*b* and 53*b*.

They may be found discussed in the books of Terroine & Zunz and of Lipschütz. LeBreton & Schaeffer, however, in their work on the nucleoproteins of the chick embryo, were led to regard the paraplasmatic substances as mainly proteins from the fact that the nucleoplasmic ratio, chemically determined, fell with age in the chick embryo. Other proteins were therefore rising, and the active mass, they thought, could be represented by (though of course it was not regarded as identical with) the nucleic acid nitrogen as percentage of the total nitrogen. Some typical figures for the chick embryo were:

| Days of development | Chemical nucleoplasmic ratio (Nucleoprotein nitrogen × 100)/(Total nitrogen − nucleoprotein nitrogen) |
|---|---|
| 8 | 10·7 |
| 10 | 8·8 |
| 13 | 6·65 |
| 16 | 4·9 |
| 20 | 3·5 |

and naturally LeBreton & Schaeffer emphasised the similarity between the fall in nucleoplasmic ratio and the fall in metabolic rate. Cahn, working under their influence, found in a study of atrophy in muscle during starvation that the nucleoplasmic ratio changed. Normal muscle contained for 100 gm. of total protein 0·684 gm. of nucleic acid, but atrophied muscle 0·961 gm. of nucleic acid for every 100 gm. of total protein. This was regarded as support for the theory of paraplasmatic substances, but the interpretation has been much criticised by Terroine. Terroine & Ritter carried out experiments which were the converse of those of LeBreton and Schaeffer on nucleic acid, for, instead of taking the same organism at different ages (and therefore sizes), they took different organisms of various sizes. Their results worked out thus:

| | Purine nitrogen in grams per 100 gm. wet weight of tissue | |
|---|---|---|
| | Muscle | Liver |
| Ox ... ... ... | 0·061 | 0·146 |
| Horse ... ... ... | 0·079 | 0·125 |
| Pig ... ... ... | 0·074 | — |
| Sheep ... ... ... | 0·077 | 0·135 |
| Dog ... ... ... | 0·062 | 0·155 |
| Rabbit... ... ... | 0·078 | 0·148 |
| Hen ... ... ... | 0·071 | 0·150 |
| Pigeon ... ... | 0·108 | 0·147 |
| Rat ... ... ... | 0·076 | 0·160 |

They concluded that these animals contained almost exactly the same amounts of nucleic acid in their cells, and that there was

no relation between heat-production and this factor, but as they neglected to estimate the total nitrogen, and so did not calculate the chemical nucleoplasmic ratio, their figures cannot be compared directly with those of LeBreton & Schaeffer for change with age.

Then Moulton's figures on the cow for nitrogen content of whole animals have often been taken as evidence in favour of a direct relation between surface and total protein, but for small animals, Terroine, Brenckmann & Feuerbach could not find such a condition. Terroine, in his review of the subject, considers various other possible measures of the active mass, such as the work of Mayer & Schaeffer on lipoids, and that of Lapicque & Petetin on mineral constituents. He is, of course, concerned throughout to show that, as far as the evidence goes, the composition of all animals is practically the same, and cites with special approval the investigations of Abderhalden, Gigon & Strauss, of Osborne & Jones and of Osborne & Heyl, who found very similar amino-acid distributions in proteins from a mammal, a bird, a fish and a mollusc. Nevertheless, such arguments are not at all convincing, for they essentially consist in minimising the differences which have been found to exist, and it would be equally justifiable in the present state of our knowledge to emphasise small variations just as much. The fact is that we cannot as yet be sure on the ground of the chemical analyses we have whether protoplasms of different animals are different or the same, and, until a great deal more work has been done,

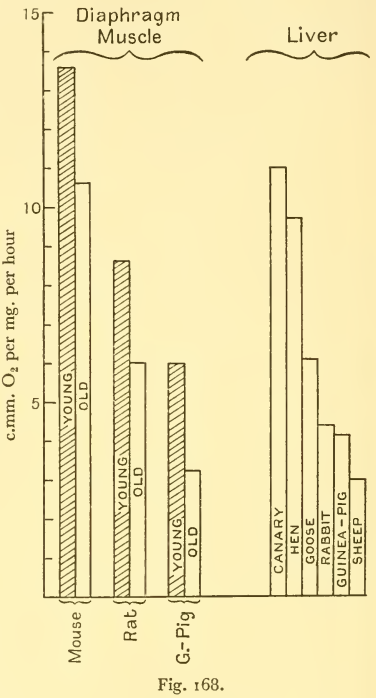


Fig. 168.

speculation will remain unprofitable. At the same time, it is quite legitimate to adopt an interim belief, either with Rubner; Richet; and Terroine, that they are the same, or with Pfaundler; Benedict; and LeBreton, that they are different. Certainly all the evidence from chemical embryology supports the latter view.

### 4·19. Respiratory Intensity of Embryonic Cells *in vitro*

Obviously the only way to decide whether surface is the governing factor in heat-production is to remove the surface, and to see whether the heat-production is then the same. This can be done by experiments on respiration *in vitro*. If *in vitro* protoplasms respire to a very similar intensity, irrespective of the size and surface of the animal from which they are derived, Terroine will be right, but if not, then LeBreton's thesis will be justified. Unfortunately, results are not unanimous. In 1924 an *in vitro* difference between large and small animals was discovered by Meyerhof & Himwich, and denied by Gräfe. In the following year, the former workers were supported by Wels, who found that, without exception, the bigger and older the animal the slower was the *in vitro* respiration. "Eine bestimmte, vom Nervensystem unabhängige Energiewechselgrösse des Gewebes zu den fundamentalen Arteigenschaften gehört", said Wels. He found, however, that birds had a higher metabolic rate than mammals of equal weight. Fig. 168 constructed from his figures shows the relationships he found. Very similar work was done by LeBreton & Kayser, who got the following figures:

| | C.c. oxygen taken up per 100 gm. wet weight per hour, at 37° |
|---|---|
| Canary ... ... ... ... | 640·7 |
| Mouse ... ... ... ... | 516·7 |
| Rat ... ... ... ... | 343·2 |
| Guinea-pig ... ... ... | 253·0 |
| Dog ... ... ... ... | 202·7 |

Roche & Siegler-Soru, thinking that blood would take up oxygen in a more normal manner in the Barcroft apparatus than pieces of tissue, obtained the following figures for the autorespiration of blood:

| Non-nucleated corpuscles | Oxygen taken up per 100 c.c. blood per hour | Calories produced per kilo per hour |
|---|---|---|
| Horse ... ... | 1·1 | 0·5 |
| Cow ... ... ... | 1·26 | — |
| Pig ... ... ... | 1·99 | 0·8 |
| Sheep ... ... | 2·07 | — |
| Dog ... ... ... | 2·15 | 2·5 |
| Rabbit ... ... | 2·72 | 3·5 |
| Guinea-pig ... ... | 2·98 | 6·0 |

| Nucleated corpuscles | Oxygen taken up per 100 c.c. blood per hour | Calories produced per kilo per hour |
|---|---|---|
| Turkey ... ... | 2·86 | — |
| Goose ... ... | 3·05 | 3·5 |
| Duck ... ... | 3·03 | 5·0 |
| Hen ... ... ... | 3·22 | 5·0 |
| Pigeon ... ... | 3·78 | 10·0 |

Then Lussana found in the embryo, with its large relative surface, a more intense respiration than in the mother, using the liver and muscle of the guinea-pig, rabbit and goat.

Table 88. *Lussana's* in vitro *experiments.*

Cubic centimetres of gas given off or taken up per 100 gm. wet weight per hour.

| | Foetus at term | | | | Maternal organism | | | |
|---|---|---|---|---|---|---|---|---|
| | Liver | | Muscle | | Liver | | Muscle | |
| | Oxygen | Carbon dioxide | Oxygen | Carbon dioxide | Oxygen | Carbon dioxide | Oxygen | Carbon dioxide |
| Guinea-pig | 1003 | 1060 | 205 | 547 | 896 | 918 | 214 | 275 |
| Rabbit | 2686 | 2707 | 283 | 315 | 2365 | 2424 | 237 | 273 |
| Dog ... | 710 | 1065 | 603 | 835 | 530 | 882 | 833 | 866 |

Better figures are those of Kayser, LeBreton & Schaeffer, who compared the oxygen uptake of various tissues according to age, as follows:

Table 89.

| White Leghorn hens. Embryo | % water | Oxygen taken in (cubic centimetres per hour at 37°) Per 100 gm. dry weight | Per 100 gm. of protein |
|---|---|---|---|
| 4 days ... | 94·53 | 1651 | 2158 |
| 5 days ... ... | 94·52 | 1090 | 1546 |
| 7 days ... ... | 94·01 | 940 | 1380 |
| 8 days ... ... | 93·45 | 747 | 1118 |
| After hatching | | | |
| 38-day brain ... | 80·15 | 1266 | 2058 |
| 2-year brain ... | 77·14 | 823 | 1543 |
| 38-day muscle ... | 76·31 | 650 | 734 |
| 2-year muscle ... | 67·71 | 226 | 249 |
| Pigeons. After hatching | | | |
| 21-day brain ... | 85·13 | 1183 | 1857 |
| 1-year brain ... | 79·83 | 1035 | 1739 |
| 21-day muscle ... | 78·65 | 559 | 791 |
| 1-year muscle ... | 61·85 | 302 | 406 |
| Rats. After birth | | | |
| 5-day brain ... | 88·57 | 1024 | 1517 |
| 200-day brain ... | 77·68 | 890 | 1428 |
| 5-day muscle ... | 86·38 | 812 | 1075 |
| 200-day muscle | 74·50 | 391 | 443 |

These figures demonstrate conclusively that the fall in metabolic rate, which is evident in the case of the intact organism, can also be seen when its tissues are considered in isolation, and can hardly therefore be due to the falling relative surface.

The fact that the oxygen consumption related to 100 gm. of protein behaved in the same way as when related to weight was taken by these authors as evidence in favour of their contention that there exist in the cell "albumines de réserve", or paraplasmatic proteins, and therefore that the total nitrogen cannot be regarded as representing the active protoplasmic mass. They regarded the demonstration of LeBreton & Schaeffer, that the chemical nucleoplasmic ratio decreased during development, as a further support for that view. The more paraplasmatic protein present, the less the nucleoprotein in proportion. Finally, they adduced the decreasing water-content which seems to be so universal an accompaniment of the growth and ageing of protoplasm (cf. Ruzicka's "law of protoplasmic hysteresis") as tending in the same direction. As they pointed out, 1 gm. of dry substance is dissolved or dispersed in 17·2 gm. of water in the 4th day chick embryo, in 3·2 gm. of water at the time of hatching, and in only 2·1 gm. in the adult hen.

Terroine & Roche, on the other hand, investigating the *in vitro* respiration of tissues, got results which differed from those of Kayser, LeBreton & Schaeffer. They did not concern themselves with the same organism at different ages, but with different organisms of various sizes (homologous tissues of homoiotherms). Their figures were as follows:

Table 90.

| Animal | Calories produced by intact animal per kilo per hour | Cubic millimetres of oxygen taken up *in vitro* per gram per hour (dry weight) | | | |
|---|---|---|---|---|---|
| | | Muscle | Liver | Brain | Kidney |
| Rabbit ... ... | 4 | 1611 | 1645 | — | — |
| Chick ... ... | 5 | 1603 | 1484 | — | — |
| Guinea-pig ... | 6 | 1636 | 1396 | 2526 | 1715 |
| Pigeon ... ... | 10 | 1565 | 1509 | 2733 | 1764 |
| Mouse ... ... | 20 | 1601 | 1764 | 2669 | 2025 |
| Finch ... ... | 37 | 1532 | 1511 | 2476 | — |

There was evidently no relation at all between the *in vitro* respiration of the tissues of the different animals, in spite of their different heat-production *in vivo*. It is much to be wished that further work could be done along these lines.

## 4·20. Embryonic Tissue-respiration and Glycolysis

The study of the respiration of embryonic tissues *in vitro* has taken a great step forward through the work of Warburg and his collaborators, who have related the oxygen consumption of tissues in a very interesting way with the type of metabolism going on in them. Preliminary researches on technique by Warburg and by Minami showed that it was possible to determine on the same material the oxygen consumption per gram per hour and the amount of lactic acid produced both in air and in nitrogen. The lactic acid produced was estimated by using bicarbonate buffer solutions, and calculating from the amount of "extra carbon dioxide" given off[1]. In the first paper of the series Warburg, Posener & Negelein studied the relations between respiration and glycolysis in a number of tissues. Their basic concepts were analogous to those universally employed with regard to muscle metabolism. In the linked reactions

$$\text{Glucose} \longrightarrow \text{Lactic acid} \longrightarrow CO_2 \text{ and } H_2O$$

one of the two may be slower than the other; the oxidative power of the tissue may be able to remove the lactic acid as fast as it is formed, or conversely, oxidation may be the slower process and lactic acid will tend to accumulate. In intact muscle, as has long been known, the desmolysis and oxidation processes are controlled so that anaerobically lactic acid is formed in great amount, but aerobically it is rapidly oxidised. In chopped muscle, the desmolytic process gets out of control, and nearly as much lactic acid accumulates aerobically as anaerobically; in other words, the oxidation process cannot keep pace with it. By the study of the relative activities of these mechanisms, Warburg and his associates were able to classify tissues to a considerable extent. In this work they used three symbols, defined as follows:

$$Q_{O_2} = \frac{\text{c.mm. of } O_2 \text{ used up}}{\text{mgm. of tissue} \times \text{hours}} \text{ (respiration) R.R.}$$

$$Q^{O_2}_{CO_2} = \frac{\text{c.mm. extra } CO_2 \text{ given out in } O_2}{\text{mgm. of tissue} \times \text{hours}} \text{ (aerobic glycolysis) O.G.R.}$$

$$Q^{N_2}_{CO_2} = \frac{\text{c.mm. total}^2\ CO_2 \text{ given out in } N_2}{\text{mgm. of tissue} \times \text{hours}} \text{ (anaerobic glycolysis) N.G.R.}$$

[1] Assuming the R.Q. of the tissue to be unity.

[2] Anaerobically there can be no "extra" $CO_2$ because respiration in the sense of oxygen-consumption is not proceeding; all of it must be due to lactic acid formation.

For convenience the three symbols adopted here will be R.R. (respiratory rate), O.G.R. (aerobic glycolysis rate), and N.G.R. (anaerobic glycolysis rate). 1 c.mm. of extra carbon dioxide (from the bicarbonate) corresponds to 0·004 mgm. of lactic acid. Warburg, Posener & Negelein made many experiments to delimit accurately the effect of *p*H, concentration of bicarbonate buffer and glucose, temperature, presence of serum, etc., on the three entities: thus with increasing *p*H (6·7 to 7·8) the N.G.R. rose from about 7 to about 16. But what more directly interests us is their comparative results for different tissues, in the estimation of which they were careful to observe standard conditions (37·5°, 0·2 per cent. glucose, $2{\cdot}5 \times 10^{-2}$ % bicarbonate, *p*H 7·66). They noted here yet a further entity, namely, the Meyerhof-quotient $\frac{\text{lactic acid disappearing}}{\text{respiration}}$ or $\frac{\text{N.G.R.} - \text{O.G.R.}}{\text{R.R.}}$ (M.Q.), which gives a measure of the extent to which the lactic acid formed is built up again into the glucose or the hexose-phosphate.

The results of their experiments are given in Table 91. A glance at it shows several very important results. In the first place, the Meyerhof-quotient is normal for all tissues studied, a fact which Warburg regarded as evidence that the desmolysis mechanism, though working unduly intensely in certain tissues (especially the neoplasms), was normal in its nature. In every case, moreover, the rate of desmolysis of carbohydrate, or rather the rate of accumulation of the lactic acid, is higher anaerobically than aerobically, but tissues vary a great deal in the extent to which this is so. In some cases, the percentage inhibition of lactic acid accumulation which occurs when the tissue passes from anaerobic to aerobic conditions is great, perhaps as much as 95 per cent. But in other cases, and these include the neoplasmatic tissues, the inhibition due to the letting in of oxygen is only small. Thus the rat carcinoma has a glycolytic power 124 times that of adult blood, 200 times that of resting frog muscle, and 8 times that of acting frog muscle. As for the respiratory intensity, it varies quite independently of the other entities, and is sometimes large, sometimes small. As the extreme instance of the glycolytic tissue, Warburg cited yeast, which desmolyses large amounts of sugar aerobically as well as anaerobically. As the extreme instance of the respiratory tissue, there is Pasteur's mould, *Mucor mucedo*, and the table provides an example, rabbit pancreas or submaxillary gland, for instance, allowing no lactic acid at all to accumulate aerobically. It was obviously

very important that Warburg found the tissue of neoplasms to behave unlike muscle and like the yeast-cell. But it is more interesting for the present purpose to note that he found the chick embryo (from the 3rd to the 5th day of development) to be different alike from adult and from neoplasmatic tissue. In its efficiency at removing lactic acid when allowed air, it resembled muscle, but its general metabolic level was of course higher and appeared in the big R.R. The relation between aerobic glycolysis (O.G.R.) and R.R. is also interesting; thus from the last column it appears that the chick embryo produces only 0·1 mol. of lactic acid for every mol. of oxygen taken in—a very different state of affairs from the tumour-cells, which will produce as much as 3·9 mol. of lactic acid for every mol. of oxygen, though it is not unlike the adult tissues, which occupy an intermediate position. Warburg found that by adding a trace of hydrocyanic acid to the medium containing the embryonic tissues he could, as it were, put a spoke into the wheels of the oxidation mechanism, and bring about a state of affairs resembling that of tumours. Thus he was able to send up the O.G.R. of the 4th-day chick embryo from 1·1 to 12·0, to bring down the percentage inhibition from 96 to 45, and to make the embryo produce 3·4 mol. of lactic acid for every mol. of oxygen taken in. This was a good imitation of a malignant carcinoma. Moreover, tumour-cells + hydrocyanic acid gave the same O.G.R. as N.G.R., showing that oxidations had been entirely depressed. The benign tumour-cells, with their O.G.R./R.R. of about 0·9 could, he found, be equally well imitated by incubating embryonic tissue anaerobically for some time before beginning the experiment. But though he was able thus to induce in embryonic material the characteristics of neoplasmatic tissues, he was not able to reverse the process, or to ascertain how it was that these characteristics were retained for a great length of time by some cells. Such observations as these acquire no little significance from the fact that sarcomata can be produced in adult animals by injecting embryo pulp with arsenious acid (Carrel; White; Askanasy; McJunkin & Cikrit; denied by Begg & Cramer) with tar (Carrel) and with indol (Carrel).

The metabolism of the neoplasms was called by Warburg that of disorganised growth, and the metabolism of the embryo that of organised growth. These distinctions are of interest in view of the work of Byerly (see p. 607), and have a relation to much recent

Table 91.

| Material | Respiratory rate (R.R.) | Aerobic glycolysis rate (O.G.R.) | Fermentation excess (U.) (N.G.R. − 2 R.R.) | Anaerobic glycolysis rate (N.G.R.) | Lactic acid in % of dry weight of tissue/hour | % inhibition of glycolysis by oxygen | Meyerhof quotient $\left(\frac{\text{N.G.R.} - \text{O.G.R.}}{\text{R.R.}}\right)$ | Warburg quotient $\left(\frac{\text{O.G.R.}}{\text{R.R.}}\right)$ | Investigator | |
|---|---|---|---|---|---|---|---|---|---|---|
| Rous hen sarcoma | 5·0 | — | +20 | 30 | — | — | — | — | Warburg, Posener & Negelein | |
| Jensen rat sarcoma | 9·0 | — | +16 | 34 | — | — | — | — | | |
| Malignant: | | | | | | | | | | |
| Flexner-Jobling rat carcinoma | 7·2 | 25 | +17 | 31 | — | 23 | 1·3 | 3·9 | ,, | ,, |
| Human epithelioma | 5·1 | 14 | — | 21 | — | 34 | 1·7 | 3·1 | ,, | ,, |
| Human sarcoma | 4·9 | 16 | +18 | 28 | — | — | 2·5 | 3·2 | ,, | ,, |
| Benign: | | | | | | | | | | |
| Human tumours | 13 | 16 | — | 26 | — | 37 | 0·8 | 1·2 | ,, | ,, |
| Human nasal polypi | 5·2 | 4·6 | +4 | 14 | — | 68 | 1·9 | 0·9 | ,, | ,, |
| Human lymphadenoma | 9·3 | 9·2 | — | 18 | — | 48 | 1·2 | 0·94 | ,, | ,, |
| Chick embryos: | | | | | | | | | | |
| 3 days | — | — | — | {26·5 / 18·5} | — | — | — | — | ,, | ,, |
| 3·5 ,, | — | — | — | 20·6 | — | — | — | — | ,, | ,, |
| 4 ,, | — | — | — | {27·8 / 20·7} | — | — | — | — | ,, | ,, |
| 5 ,, | — | — | — | 18·2 | — | — | — | — | ,, | ,, |
| Average | 10 | 1·1 | 0 | 23 | — | 96 | 2·0 | 0·1 | ,, | ,, |
| Plus hydrocyanic acid | 3·6 | 12 | — | 20·6 | — | 45 | 2·2 | 3·4 | ,, | ,, |
| Incubated *in vitro* anaerobic | 7·2 | 6·8 | — | 20·6 | — | — | 1·9 | 0·94 | ,, | ,, |
| Adult tissues (stationary): | | | | | | | | | | |
| Rat intestine | 12·0 | — | −20 | 4·0 | — | — | — | — | ,, | ,, |
| Rat liver | 11·6 | 0·6 | −21 | 3·3 | — | — | — | — | ,, | ,, |
| Rat kidney | 21 | 0 | −39 | 3·2 | — | — | — | 0·05 | ,, | ,, |
| Dog pancreas | 3·2 | 0 | − 2 | 4·2 | — | — | — | — | ,, | ,, |
| Rabbit pancreas | 4·6 | 0 | − 7 | 3·4 | — | — | — | — | ,, | ,, |
| Rabbit submaxillary gland | 4·1 | 0 | − 5 | 3·3 | — | — | — | — | ,, | ,, |
| Rat thyroid | 13 | 0 | −24 | 2·1 | — | — | — | — | ,, | ,, |

Table 91 (*contd.*).

| Material | Respiratory rate (R.R.) | Aerobic glycolysis rate (O.G.R.) | Fermentation excess (U.) (N.G.R. − 2 R.R.) | Anaerobic glycolysis rate (N.G.R.) | Lactic acid in % of dry weight of tissue/hour | % inhibition of glycolysis by oxygen | Meyerhof quotient $\left(M.Q.\right) = \frac{N.G.R. - O.G.R.}{R.R.}$ | Warburg quotient $\left(\frac{O.G.R.}{R.R.}\right)$ | Investigator |
|---|---|---|---|---|---|---|---|---|---|
| Adult tissues (not stationary): | | | | | | | | | |
| Rat thymus | 5·8 | 0·6 | − 4 | 8·2 | — | — | — | 0·1 | Warburg, Posener & Negelein |
| Human lymph gland | 3·8 | 2·2 | — | 4·7 | — | — | — | 0·58 | |
| Human tonsil | 5·1 | 3·4 | 0 | 12·8 | — | — | — | 0·66 | ,, ,, |
| Rat testis | 12·3 | 7·2 | −16 | 8·5 | — | — | — | 0·59 | ,, ,, |
| Resting frog muscle | — | 0 | — | 1·5 | — | — | — | — | ,, ,, |
| Rat cerebral grey matter | 10·7 | 2·5 | − 3 | 19·1 | — | 87 | 1·6 | 0·2 | ,, ,, |
| Rat retina | 30·7 | 45 | +26 | 88 | — | 49 | 1·4 | 1·5 | ,, ,, |
| Frog retina | 3·5 | 0·66 | — | 9·5 | — | — | — | 0·19 | Negelein |
| Rat spleen | 12·0 | — | −16 | 8·0 | — | — | — | — | Murphy & Hawkins |
| Rat placenta | 7·3 | — | + 0·3 | 14·9 | — | — | — | — | ,, ,, |
| Yeast | 96 | — | — | 192 | — | — | — | 0·5 | Warburg |
| Rat embryos (dry weight): | | | | | | | | | |
| 0·5 mgm. | — | 0 | — | 35 | 14 | — | — | — | Negelein |
| 1·0 ,, | 13 | 0 | − 3 | 22 | 8 | — | — | — | ,, |
| 2·0 ,, | — | 0 | — | 15 | 6·8 | — | — | — | ,, |
| 3·0 ,, | 12 | 0 | −11 | 12·5 | 4 | — | — | — | ,, |
| 4·0 ,, | — | 0 | — | 11 | 3·8 | — | — | — | ,, |
| 5·0 ,, | — | 0 | — | 10 | 3·8 | — | — | — | ,, |
| 6·0 ,, | — | 0 | — | 9 | — | — | — | — | ,, |
| 7·0 ,, | — | 0 | — | 8 | — | — | — | — | ,, |
| 8·0 ,, | — | 0 | — | 7 | — | — | — | — | ,, |
| 9·0 ,, | — | 0 | — | 6 | — | — | — | — | ,, |
| 10·0 ,, | — | 0 | — | 5 | 3·0 | — | — | — | ,, |
| Chorionic membranes, embryo (dry weight): | | | | | | | | | |
| 0·5 mgm. | — | — | — | 48 | 19·1 | — | — | — | ,, |
| 6·0 ,, | — | — | — | 43 | 17·4 | — | — | — | ,, |
| 12·0 ,, | — | — | — | 38 | 14 | — | — | — | ,, |
| 18·0 ,, | — | — | — | 35 | — | — | — | — | ,, |
| 24·0 ,, | 18 | 0 | − 3 | 32·5 | — | — | — | — | ,, |
| Retina, adult hen | 0·4 | 134 | — | 137 | — | 0·5 | — | — | Krebs |
| Adult dove | 0·4 | 180 | — | 180 | — | — | — | — | ,, |
| Chick embryo: | | | | | | | | | |
| Retina, 16 days' incubation | 10·6 | 33 | — | 54 | — | — | 2·0 | — | ,, |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Chick embryo: | | | | | | | | | | |
| Retina, 8 days | — | — | — | 35 | — | — | — | — | Tamiya | |
| 15 ,, | — | — | — | 45 | — | — | — | — | ,, | |
| 20 ,, | — | — | — | 85 | — | — | — | — | ,, | |
| 1 year after hatching | — | — | — | 130 | — | — | — | — | ,, | |
| 4 years ,, | — | — | — | 90 | — | — | — | — | ,, | |
| Liver (dry weight): | | | | | | | | | | |
| 0·05 mgm. | — | — | — | 20 | — | — | — | — | ,, | |
| 0·15 ,, | 15·4 | 0 | −20·1 | 13 | — | — | — | — | ,, | |
| 0·25 ,, | 13·7 | 0 | 19·4 | 11 | — | — | — | — | ,, | |
| 0·9 ,, | 12·1 | 0 | −16·2 | 8 | — | — | — | — | ,, | |
| 2·25 ,, | — | — | — | 6 | — | — | — | — | ,, | |
| (Adult) 8000·0 ,, | 14·4 | 0 | −26·7 | 3 | — | — | — | — | ,, | |
| Fibroblasts in culture | 14·0 | 4 | − 3·5 | 17 | — | — | — | — | Warburg & Kubowitz | |
| Chick embryo: | | | | | | | | | | |
| Epithelial cells | 24·6 | 12·5 | −15 | 34 | — | — | — | — | ,, | ,, |
| Heart, 4 days | 30 | 12·5 | − 8·1 | 52 | — | — | — | — | ,, | ,, |
| 6 ,, | 14·6 | 0 | − 4·2 | 25 | — | — | — | — | ,, | ,, |
| 7 ,, | 15·1 | 3·3 | −16 | 15 | — | — | — | — | ,, | ,, |
| Chick embryo (dry weight): | | | | | | | | | | |
| 0·27 mgm. | 22·4 | 5·5 | −27 | 18·5 | — | — | — | — | Kumanomido | |
| 0·905 ,, | 14·3 | 0·6 | −17 | 11·4 | — | — | — | — | ,, | |
| 4·73 ,, | 8·1 | 1·2 | − 8·8 | 7·5 | — | — | — | — | ,, | |
| Rat placenta | 7·3 | 10·6 | + 0·3 | 14·9 | — | — | 0·83 | 1·5 | Murphy & Hawkins | |
| Human placenta: | | | | | | | | | | |
| 9th month whole | 2·05 | 0·5 | + 0·5 | 4·6 | — | — | — | — | Bell, Cunningham, Jowett, Millet & Brooks | |
| (Chorionic epithelium removed) | 4·0 | — | − 2·6 | — | — | — | — | — | | |
| Human placenta, whole: | | | | | | | | | | |
| 3rd month | 5·12 | 2·53 | — | 6·07 | — | — | — | — | Loeser | |
| 9th ,, | 2·33 | 1·68 | — | 5·59 | — | — | — | — | ,, | |
| Rat embryo, lung | 10 | 0 | — | 14 | — | — | — | — | Fujita | |
| Rat embryo, lens | 8 | 17 | — | 27 | — | — | — | — | ,, | |
| Rat placenta | 7 | 3 | — | 15 | — | — | — | — | ,, | |
| Rat liver: | | | | | | | | | | |
| Embryo | 7·1 | 0·5 | − 4·9 | 9·25 | — | — | 1·23 | 0·075 | Rosenthal & Lasnitzki | |
| Shortly after birth | 15·0 | 0 | −24 | 3·0 | — | — | — | — | ,, | ,, |
| Adult | 11·3<br>14·1 | 0·8<br>1·13 | −21·5<br>— | 1·14*<br>2·33† | — | — | — | 0·073<br>0·08 | ,, | ,, |
| Rat kidney: | | | | | | | | | | |
| Embryo | 13·8 | 0·85 | −10·2 | 17·4 | — | — | 1·17 | 0·06 | ,, | ,, |
| Shortly after birth | 19·7 | 0 | −34·2 | 7·0 | — | — | — | — | ,, | ,, |
| Adult | 19·0<br>32·7 | 0<br>0 | −34·7<br>— | 3·27*<br>2·73† | — | — | — | — | ,, | ,, |
| Mouse placenta (ripe) | 4·2 | 3·2 | — | 7·8 | — | — | — | — | Adler | |
| Rat ,, ,, | 1·2 | 4·7 | — | 11·1 | — | — | — | — | ,, | |
| Guinea-pig ,, ,, | 0·9 | 0·6 | — | 3·7 | — | — | — | — | ,, | |
| Man | 0·7 | 1·7 | — | 3·0 | — | — | — | — | ,, | |

investigation of differentiation-processes. Referring again to Table 91, it will be found that embryonic tissue has a very considerable lactic acid production in anaerobic conditions, as much as that of the neoplasms, but that it differs from them on the one hand by the efficiency of its aerobic oxidation mechanism (40 times as adequate as that of rat carcinoma), and from adult tissues, on the other hand, because of their feeble anaerobic glycolysis rate. Per hour per milligram wet weight the 4th day embryo can produce anaerobically 0·09 mgm. of lactic acid, i.e. 9 per cent. of its weight; this may be set against the performance of frog's muscle (intact and anaerobic) which produced only 0·06 per cent. of its weight when resting, and 1·5 per cent. when stimulated. Warburg concluded that the high N.G.R. was a general property of growing tissues, but that the O.G.R. was only high if the growth was unorganised as in neoplasms, and regarded this as the most important outcome of his work. "Where growth is, glycolysis is", he said, "and where abnormal growth is there aerobic glycolysis is." As regards the adult tissues he tried, there were one or two difficulties. Those that might be considered stationary, such as liver and kidney, had very low N.G.R.'s and unmeasurably small O.G.R.'s, while those which were not quite rightly termed stationary, such as testis, had slightly higher N.G.R.'s and small O.G.R.'s. But the grey substance of the brain and the retina were found to have quite peculiar characteristics, an enormously high R.R., nearly three times that of the chick embryo, a very high N.G.R. and a high O.G.R. No satisfactory explanation was or is available for this curious state of affairs, but it has its importance for embryological studies, since in the earlier periods of development the brain vesicles and the eyes make up so significant a proportion of the growing body. The possibility that these results might explain some of those obtained by Child and his school must not be forgotten. Subsequently Negelein found quite different results with amphibian retina, and suggested that such a delicate tissue had been giving cytolysis results in the earlier work. But more recent researches by Krebs have confirmed it.

Finally Warburg, Posener & Negelein gave some interesting data on the subject of ammonia production, glycolysis and tissue respiration. They found that, when the tissues were placed in Ringer solution to which glucose had not been added, notable amounts of ammonia were formed, indicating a combustion of protein substances. They

compared the activities of various tissues in this direction, obtaining the following results:

| | Cubic millimetres of ammonia produced (in glucose-free Ringer)/milligrams of tissue × hours | N.G.R. |
|---|---|---|
| Rat thyroid ... ... | 0 | 2 |
| Rabbit submaxillary ... | 0·03 | 3 |
| Rat liver ... ... ... | 0·07 | 3 |
| Rabbit pancreas ... ... | 0·11 | 3 |
| Rat thymus ... ... | 0·31 | 8 |
| Rat testis ... ... ... | 0·23 | 8 |
| Chick embryo (5th day) ... | 0·56 | 23 |
| Rat carcinoma ... ... | 0·9 | 31 |
| Rat grey matter ... ... | 1·4 | 19 |
| Rat retina ... ... ... | 1·2 | 88 |

It is evident that all the tissues concerned prefer to combust carbohydrate if they can get it, but if not they will combust protein. It would be very interesting to know how this property varies during development in the chick. A certain parallelism is to be observed between the anaerobic glycolysis rate (not the R.R.) and the ammonia-production rate in aerobic glucose-free conditions. The addition of glucose abolished the ammonia production both aerobically and anaerobically.

The next important paper was that of Negelein, who devoted himself particularly to the examination of the changes taking place in respiratory characteristics during embryonic growth. Chick embryos, as Warburg, Posener & Negelein had already found, produced anaerobically 9 per cent. of their weight in lactic acid per hour; rat embryos, Negelein now found, produced only between 3 and 7 per cent. But all depended on the time of development, for in the earlier stages rat embryos had a figure of about 14 per cent. The amniotic membranes and the chorion were found to have an even larger figure, as much as 19·6 per cent., i.e. fully as high an N.G.R. as neoplasms. The curve which resulted from Negelein's observations appears in Fig. 169. The measurements were nearly all

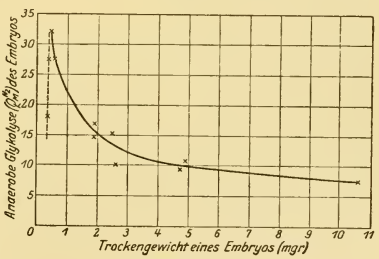


Fig. 169.

done in inactivated horse serum, though in later stages amniotic liquid was used. For comparison some of the figures are included in Table 91. The curve obviously declines with increasing age, the N.G.R. falling from about 35 to about 5, so that, as can be seen from Table 91, the N.G.R. declines during the embryonic period of the rat from a value equivalent to that of malignant neoplasms and to the very young chick embryo to a value very close to that of the various adult rat tissues. According to one or two of the earliest measurements of Negelein, there is a possibility that the high value of 35 at 0·5 mgm. dry weight may be a peak, to which the curve has risen from earlier lower values. If this turns out to be the case, there will be an interesting parallel with the hen's egg, which, according to Tomita's observations, has a marked peak in lactic acid content at the 5th day of incubation (see Fig. 292). Negelein argued that the unfertilised egg-cell has probably only a very small N.G.R., so that a peak would be expected. That it comes so early in development is a fact of importance from the point of view of the energy source used by the embryo. Negelein found that the N.G.R. of the chorionic membranes of the rat embryo declined in much the same way as that of the embryo itself. This is shown in Fig. 170, where the age of the membranes, expressed in embryo dry weights, is plotted against the N.G.R., and a few figures are given in Table 91. It is noteworthy that the N.G.R. of the membranes is always much higher than that of the embryo, although it falls in unison with it, if a little slower. It maintains its N.G.R. at a level well above that of most neoplasms, so that its hydrolytic mechanisms must be exceedingly powerful. Negelein only did one experiment with the amniotic membrane separately; it gave an N.G.R. of 35·8 at an embryo dry weight of 60 mgm., i.e. slightly above the corresponding N.G.R. of the chorion.

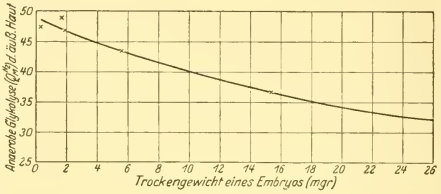


Fig. 170.

Negelein's rat embryos gave rather variable results with respect to the O.G.R., for in Ringer solution a measurable amount of lactic acid was formed aerobically, but in serum this only occurred in the

early stages. Negelein's conclusion was that in the best conditions there was practically no O.G.R., a finding which agreed very well with the previous results of Warburg, Posener & Negelein on the chick embryo.

In 1927 Warburg collected together the data which had accumulated for various tissues, with the object of proposing another entity, the fermentation excess, or U., which he defined as equal to N.G.R. − 2 R.R. Previously the comparison of tissues on the basis of their O.G.R. had been difficult because of the high susceptibility of the reaction on which it depended; thus rat embryos in Ringer had a considerable O.G.R. but in serum only a very small one, while in amniotic fluid they had none at all. He therefore suggested that it would be best not to measure the O.G.R. directly, but to calculate it from theoretical grounds. U. would be 0 when the N.G.R. equalled double the R.R., and negative or positive if it was lower or higher than double the R.R. In the latter case, the oxidation mechanisms would be inferior in efficiency to the glycolytic mechanisms and *vice versâ*. Values for U. are given in Table 91.

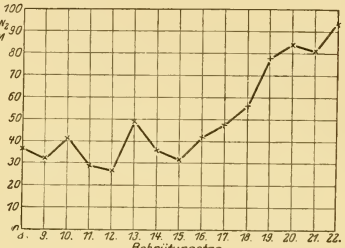

Fig. 171.

Other workers continued the investigations in the matter of embryonic tissues. Krebs discovered the interesting fact that the adult bird retina had a metabolism almost entirely composed of glycolysis, with an almost unmeasurable respiration. His figures are shown in Table 91. At the same time he found that the embryonic retina had not so extreme a type of metabolism, having an O.G.R. of 33, unlike the value of 133 for the adult. The construction of a curve relating avian retina N.G.R. to age was undertaken by Tamiya, whose graph is shown in Fig. 171. Beginning on the 8th day of development at about 35, it rose steadily during incubation to reach 45 at the 15th day and 85 at hatching, after which it continued to rise during postnatal life, until the adult value of 130 was reached. Some observations seemed to show that in old age it declined again, and was 90 at 4 years old. This result implied that just before

hatching the embryonic retina produced anaerobically 20 per cent. of its weight per hour lactic acid, but that 1 year after hatching it produced under similar conditions in the same time 65 per cent. of its own weight. This tissue has certainly a far greater glycolytic intensity than any other known, and Cramer suggests that this peculiar property may account for the fact that human epitheliomata and neuroepitheliomata of the retina have a very short induction-period unlike all other neoplasms. On the other hand the embryonic lens of the rat was shown by Fujita to have a constantly descending N.G.R. (see Fig. 172).

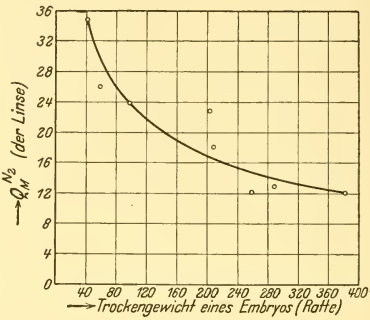


Fig. 172.

Tamiya later made further experiments on the developing liver of the chick. His graph is shown in Fig. 173. It resembles the declining glycolytic rate of the rat embryo, and differs entirely from the behaviour of the retina. U. calculated for each stage was negative,

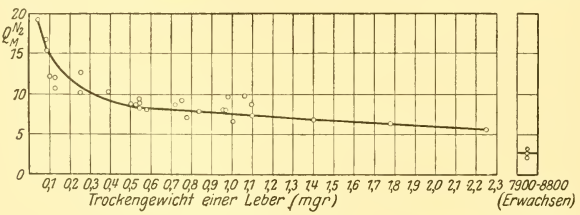


Fig. 173.

i.e. O.G.R. was very low, and the liver-cells at all times during their growth were able to deal with as much lactic acid as their desmolytic mechanism supplied them with. Their metabolism was throughout that of well-organised growth, like that of the rat embryo. Similar work was done by Warburg & Kubowitz on embryonic fibroblasts, epithelial cells and heart cells. The figures are given in Table 91. For the heart, their results agreed well with those of Tamiya for the embryonic liver. As age increased, and the weight

of the heart (dry) rose from 0·1 to 0·8 mgm., so the N.G.R. fell from about 50·25 to 14. It is interesting to note that, in their figures for tissue cultures of fibroblasts, the R.R. and the N.G.R. were both systematically higher the fewer the number of sub-cultures that had been gone through since the tissue was first explanted. Perhaps this might be regarded as evidence that with increasing age the metabolic rate declines in tissue culture, just as the growth-rate does, as was shown by Cohn & Murray (see p. 461 and Figs. 62 and 174).

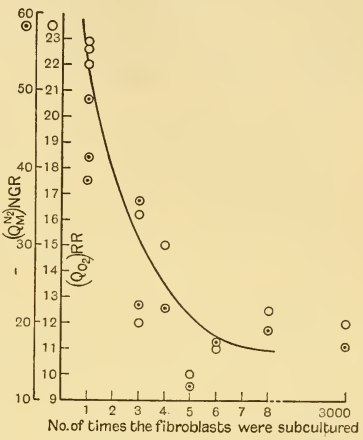


Fig. 174.

Work on embryo tissues was further extended by Kumanomido, who studied the respiratory metabolism of chick embryos in chick serum and in Ringer solution. The N.G.R. was in all cases some 50 per cent. higher in the latter than in the former. Kumanomido felt able to conclude that this difference was not due to a cytolysis or other injury effect in Ringer, but to an inhibiting effect of serum, owing to which the maximum intensity of lactic acid production was not reached there. Dialysis of the serum so that most of the salts and crystalloidal substances disappeared did not affect its depressant property, nor did heating for 30 minutes at 55°. Kumanomido did not state, however, whether these conclusions applied to the O.G.R. as well, but it would be important to know this, in view of Negelein's results on rat embryos, where there was a measurable O.G.R. in Ringer but not in serum. Fig. 175, taken from Kumanomido, shows the decline in N.G.R. with age during incubation in the chick embryo. This graph is directly comparable with that of Negelein for the pre-natal life of the rat, shown in Fig. 169 and with that of Rosenthal & Lasnitzki for the developing liver and kidney of the rat shown in Fig. 176.

The extent of the fall in anaerobic glycolysis rate does not seem to be so great in the chick as in the rat, for in the former the drop is from 27 to 10, and in the latter from 35 to 5. But Kumanomido's

chick embryos were all taken during a very short period, namely the 2nd to the 5th day of incubation, and it is possible that a more complete study would reveal a greater fall. In Table 91 it will be noted that, following the usual course, U. is always negative, and, though the N.G.R. is fairly high, the O.G.R. is low. The R.R. shows some indications of a fall with age, agreeing with other figures for metabolic rate.

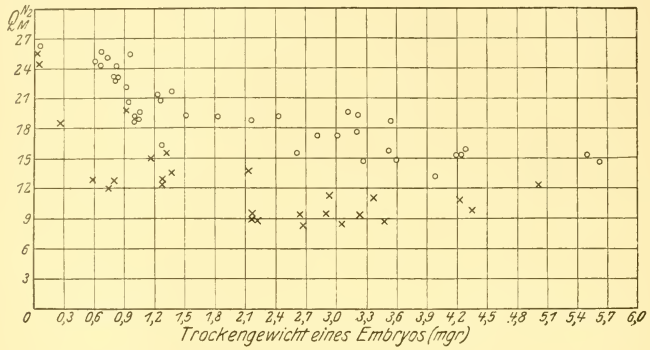


Fig. 175.

○ in Ringer solution × in avian serum

It appears that in general the N.G.R. declines with age[1]. This has been found to be true in several investigations:

| | |
|---|---|
| Rat liver ... ... | Rosenthal & Lasnitzki |
| Rat kidney ... ... | Rosenthal & Lasnitzki |
| Rat embryo (whole) ... | Negelein |
| Rat chorion ... ... | Negelein |
| Human placenta ... | Loeser |
| Chick liver ... ... | Tamiya |
| Chick heart (*in vitro*) ... | Warburg & Kubowitz |
| Chick embryo (whole) ... | Kumanomido |
| Rat lens and placenta ... | Fujita |
| Rat placenta ... ... | Adler |

The converse, i.e. that the N.G.R. rises with age, has only been observed in one case:

| | |
|---|---|
| Chick retina ... ... | Tamiya |

The aerobic glycolysis rate, on the contrary, may either rise or fall with age:

| | |
|---|---|
| Rise: Rat liver ... ... | Rosenthal & Lasnitzki |
| Fall: Human placenta ... | Loeser |
| Chick embryo (whole) ... | Kumanomido |

[1] Fertilisation, according to Ashbel, augments the N.G.R. of echinoderm eggs.

It would seem, therefore, that Hawkins is not very wide of the mark when he suggests that N.G.R. is a function of growth-rate. He believes that tissues can be classified better in this way than by using R.R. and O.G.R. relationships as the German school does.

Kumanomido also made a few experiments with the chorion of hen and rat embryos and with rat embryos themselves, finding in all cases that the N.G.R. was higher in Ringer solution than in serum. He examined a few normal human chorionic membranes and placentas, and obtained a very low N.G.R. (of about 5). There was here a certain contradiction between his results and those of Murphy & Hawkins, who worked with rat placentas, and got the results shown in Table 91. However, neither of these papers contain details of the age of the placentas used, though from Kumanomido's description it would seem that he used full-term material. Murphy & Hawkins' paper was mostly concerned with the *in vitro* respiration and glycolysis of various types of neoplasm, but they confirmed the results of Warburg, Posener & Negelein on the chick embryo, and stated that the same type of respiratory metabolism was shown by rat embryo skin, rat embryo membranes, and the wall of the pregnant rat uterus, although they gave no figures in support of this.

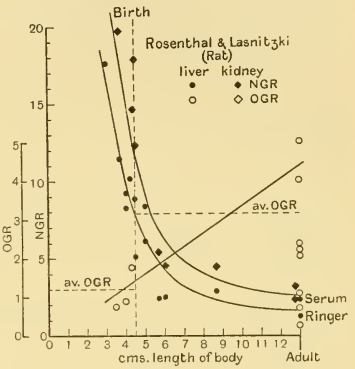

Fig. 176.

The most illuminating suggestion as regards the placenta in this connection is that of Bell, Cunningham, Jowett, Millet & Brooks, who found, as is shown in Table 91, that the early human placenta gave a positive U., i.e. possessed on the whole a more active glycolytic than oxidative mechanism. Then, removing the chorionic epithelium, they made the experiment again, and found that the result was now a negative U. like that given by practically all non-neoplasmic tissues. They therefore did not hesitate to regard the penetration of the maternal tissue by the invading foetal trophoblast as truly "malignant", a standpoint of much interest from several points of view

(see also Superbi). Thus Hammond who implanted foetal tissues into the uterine wall, found that they possessed no power of establishing themselves there, unlike the foetal trophoblast. The uterine grafts were absorbed.

Summarising, we may say that four main types of respiratory metabolism have been revealed by the studies initiated by Warburg:

(*a*) *Normal resting tissue*—anaerobic glycolysis slight, aerobic glycolysis absent, respiratory rate generally rather high, U. negative, Warburg quotient $\leqq 0{\cdot}3$.

(*b*) *Normal growing tissue* (*embryonic*)—anaerobic glycolysis high, aerobic glycolysis very slight or absent, respiratory rate high, U. negative, Warburg quotient $\leqq 0{\cdot}3$.

(*c*) *Abnormal growing tissue* (*malign neoplasms*)—aerobic and anaerobic glycolysis both high, respiratory rate usually low, U. positive, Warburg quotient $> 2{\cdot}0$.

(*d*) *Abnormal growing tissue* (*benign neoplasms*)—all factors generally rather low, U. positive, Warburg quotient $0{\cdot}5$–$1{\cdot}3$.

Completer details are to be found in the reviews of Warburg & Minami; Warburg, Negelein & Posener; Cramer; Cannan; and particularly Warburg.

### 4·21. The Genesis of Heat regulation

The only other subject which calls for consideration in this chapter is that of the ontogeny of heat regulation. It has been known for a long time that, just as animals which are normally homoiosmotic are yet poikilosmotic while in their embryonic state, so the capacity of heat regulation possessed by homoiothermic organisms arises at a definite moment in the individual life-cycle.

Edwards, who worked about the year 1820, was the first to notice this. He found that the temperature of newly born puppies, kittens, and rabbits fell when the animal was removed from its warm surroundings, and continued to fall until it almost reached the temperature of the air. Guinea-pigs, on the other hand, were able to maintain their temperature very well immediately after birth. Edwards divided homoiothermic animals into two classes, (*a*) those which are at birth blind, helpless, naked, and poikilothermic, and (*b*) those with open eyes, skin covered with hair or feathers, and homoiothermic. This classification corresponds exactly with nidifugous and nidicolous birds (see pp. 272 and 317). In the case of

the dog, the cat, and the rabbit, the adult condition is reached 15 days after birth. Edwards found that these phenomena were not due to the surface/volume ratio of the small animal, and showed that skin covering had little to do with it, for an adult sparrow was able to maintain and regulate its temperature perfectly in the absence of all feathers. Many years later Raudnitz, from a study of the temperature of newly born human infants, concluded that the chief cause of variable temperature was the imperfect development of homoiothermicity.

Babák, continuing the analysis of the problem, divided heat-regulation into two types, "chemical" and "physical". The human newborn infant, for example, possesses the former function, for it can counter a fall of environmental temperature by increasing its combustions; but it cannot resist a rise and immediately goes into hyperthermia, having an imperfect control of its capillaries and its transpiration. Plant subsequently extended this point of view to other mammals such as the cat and dog.

The bird embryo was first investigated in this connection by Pembrey, Gordon & Warren. They demonstrated that, during the incubation period in the chick, changes of external temperature invariably determine, after a longer or shorter period, changes in the same direction in the respiratory exchange. The period required for response did not seem to depend on whether the shell was removed or not. On the other hand, when the recently hatched chick was examined, it reacted to temperature changes exactly as a warm-blooded animal would, a fall of 20° in external temperature raising the expired carbon dioxide to twice its previous amount in 15 minutes. The chick embryo, then, was cold-blooded up to the 19th day of incubation. The physiological transition was observed by Pembrey and his associates to take place on the 21st day of incubation during the time immediately prior to hatching. Sometimes there was an intermediate condition, transient but neutral, in which a fall of temperature did not result in a fall in carbon dioxide output, nor, on the other hand, in a rise. This intermediate condition may give way to the cold-blooded or the warm-blooded condition, according as to whether the chick is feeble or strong and healthy.

Pembrey later reported that the transition to full powers of heat regulation did not take place in the pigeon (a nidicolous bird) till well after hatching, i.e. about the 6th day of post-natal life. Giaja

was the next investigator who occupied himself with this problem. Giaja defined the "summit metabolism" as the maximum energy expenditure which the homoiothermic animal can rise to in its struggle against cold, and spoke of a "metabolic quotient" as follows:

$$\frac{\text{Summit metabolism}}{\text{Basal metabolism}} = \text{metabolic quotient.}$$

For the mouse and the rat the metabolic quotient is approximately 3·5, for birds 4·0. Before hatching, the chick embryo, according to Giaja's measurements, which were quite comparable with those of Pembrey, could be said to have a negative metabolic quotient, but by 6 hours afterwards the value of this constant was 1·9, and, 5 days after, it rose to 2·0, and at 48 days to 2·6. Giaja observed the intermediate state spoken of by Pembrey, i.e. the condition before hatching, in which the chick maintains its heat-production when the temperature is lowered but cannot raise it to compensate for a fall. Exactly the same results were obtained on the rabbit immediately after birth; its metabolic quotient, 1·3 at 12 hours, rose to 2·4 a fortnight later.

A certain further insight into the ontogeny of heat regulation is obtained by examining Fig. 83 *b*, which has already been described. It shows Brody & Henderson's work on the effect of temperature on the growth-rate of the chick embryo at different stages. The reason why the curve $X_3$ is not a curve but a straight line is, in their opinion, that between the 13th and 18th days of incubation the heat-regulating mechanism of the embryo has probably developed sufficiently to enable it to keep its body-temperature constant within the limits of 37·2° and 40·6°. Thus at this stage the chick embryo is not strictly cold-blooded. These experiments would authorise us to suppose that the development of heat regulation is not quite sudden, as it seemed at first to be from Pembrey's experiments, but the zone of temperatures within which it can accurately adjust itself widens continually as development progresses. The most recent investigations of this are those of Kendeigh & Baldwin. They state that "frequently in the literature of avian physiology and life-history, statements are to be found that the body-temperatures of nestling birds are extremely variable and similar to those of cold-blooded animals". Their own work on the house wren, *Troglodytes aëdon*, showed clearly that full temperature regulation was attained by the

time that the squabs were ready to leave the nest. They distinguished four factors in the installation of this function, (1) the change in the surface/volume ratio, (2) the development of feathers, (3) the development of an internal dissipating surface (the air-sacs) probably under nervous respiratory control, and (4) heat-production in metabolism. Of these they regarded the third as perhaps the most important.

In a later paper Pembrey found that mice and rats at birth have not attained the capacity for heat regulation. When they are naked, blind, and unable to run about, they cannot maintain a constant temperature, and cannot regulate the production of heat at low temperatures. When they are about 8 days old, they have a protecting coat of fur, and though still blind are much more active—at this stage they give some evidence of having acquired the power. At 10 days old they are as homoiothermic as the adults. Gulick's later statement that mice and rats are fully homoiothermic at birth cannot be accepted on the basis of his insufficient evidence. Exactly similar results were obtained on pigeon squabs, as has already been mentioned, so that the pigeon and the hen among oviparous animals correspond respectively to the rabbit and the guinea-pig among viviparous ones. More recently, Ginglinger & Kayser have confirmed the work of Pembrey and have linked up, as we have already seen, the genesis of heat-regulation with the peak in metabolic rate. Their results may be summarised as follows:

| | Form of heat-regulation present at birth | |
|---|---|---|
| | Chemical | Physical |
| Guinea-pig ... ... | + | + |
| Chick ... ... ... | + | + |
| Rabbit ... ... ... | + | − |
| Man ... ... ... | + | − |
| Cat ... ... ... | + | − |
| Mouse ... ... ... | − | − |
| Pigeon ... ... ... | − | − |

It may not be irrelevant here to mention, in connection with body-temperature, that the so-called "broody fever", i.e. a temperature higher than normal for sitting hens, has been shown by Simpson to be without factual basis.

## 4·22. Light-production in Embryonic Life

Heat is, of course, not the only form of energy which animals can radiate. Luminous organisms occasionally have luminous eggs as the following table shows:

| | | | |
|---|---|---|---|
| COELENTERATA | Ctenophores | *Beroë*. Segmentation-stages, not the eggs | Allman; Agassiz, A.; Peters, A. W. |
| ANNELIDS | Polychaeta | *Chaetopterus*. Larvae | Enders |
| ECHINODERMS | Ophiuroidea | Plutei | Mangold, E. |
| ARTHROPODS | Crustacea | Schizopod larvae | Trojan |
| | | Copepod nauplii | Giesbrecht |
| | Insects | Coleoptera. *Lampyris* | Harvey |
| | | *Pyrophorus* | ,, |
| UROCHORDA | | Tunicates. *Pyrosoma* | McKinnon |

The beetle's eggs in question shine before fertilisation, while still in the ovary. It would be interesting to know what component is missing from the early developmental stages of *Beroë*. In the case of *Pyrosoma*, according to McKinnon, the luminescence is due to the presence of symbiotic bacteria which are transmitted to each egg as it is laid by a special mechanism in the parent and are afterwards distributed among the blastomeres during the cleavage stages. Similar events take place in certain cephalopods which produce luminous eggs.

McKinnon also points out that other symbiotic organisms may be transferred to eggs. Thus the reinfection of the termites such as *Hylecoetus dermestoides* with fungal spores is brought about by the smearing of the spores on to the egg as it passes down the oviduct and the subsequent consumption by the larva of its own egg-case after hatching. The wood-wasp *Sirex* and the olive-fly *Dacus* are other instances of this. Moreover, the transmission of the symbiotic cellulose-fermenting yeasts from generation to generation occurs in a similar way in various beetles (the death-watch beetle, *Xestobium rufovillosum* (Staniland); *Sitodrepa panicea* (Breitsprecher)), also in a large number of homoptera (Richter) and in the rice-weevil, *Calandra oryzae* (Mansour). The whole subject has been well reviewed by Buchner.

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# SECTION 5

## BIOPHYSICAL PHENOMENA IN ONTOGENESIS

### 5·1. The Osmotic Pressure of Amphibian Eggs

A considerable number of workers have turned their attention to measurements of osmotic pressure in eggs and embryos, in the attempt to throw some light either, in the case of aquatic embryos, on the relation between the embryo and its environment, or, in the case of birds, for instance, on the relations as regards water and salts, between the different components of the ovum.

The best known part of the work has been done on the eggs of amphibia. The fundamental observation was made by Backmann & Runnström in 1909 that the osmotic pressure of frog's egg-*Breis* was very different according to the stage of their development. They obtained the following figures:

| | Δ (depression of the freezing-point) (° C.) |
|---|---|
| Ripe ovarial eggs ... ... | −0·48 |
| Fertilised eggs ... ... | −0·045 |
| Embryos of 5 days ... | −0·23 |
| Tadpoles of 20 days ... | −0·405 |
| Serum of adult ... ... | −0·465 |

From these simple observations, which have often been confirmed, all the subsequent work took its origin. They were in many ways interesting, for the Δ of ordinary pool fresh water was found to be − 0·06, so that after the eggs were shed from the oviduct they evidently adjusted their osmotic pressure to equal that of their immediate environment. Then, later, they acquired some kind of independence, and by the time of hatching were well on their way to attaining the adult osmotic pressure. The question as to how the initial fall in osmotic pressure was achieved was not easy to answer. It might be accounted for, of course, on the supposition that the eggs took up water from their hypotonic medium, and diluted their contents, but if this had been so the increase in volume would have been far greater than was actually found. Backmann & Runnström preferred to assume that the egg-colloids were rapidly gelated after the eggs were shed, and inorganic ions adsorbed on to them, a process which would certainly lower the osmotic pressure, but would

have to be reversible. Whatever the mechanism, the facts showed that in its earliest stages the frog's egg was poikilosmotic, becoming homoiosmotic as it developed; a conclusion of general interest in view of all the work reviewed in the last section, which demonstrates a passage from poikilothermicity to homoiothermicity in the case of embryos during their development.

In later papers Backmann & Runnström extended their researches at length. They found that amphibian eggs would not develop

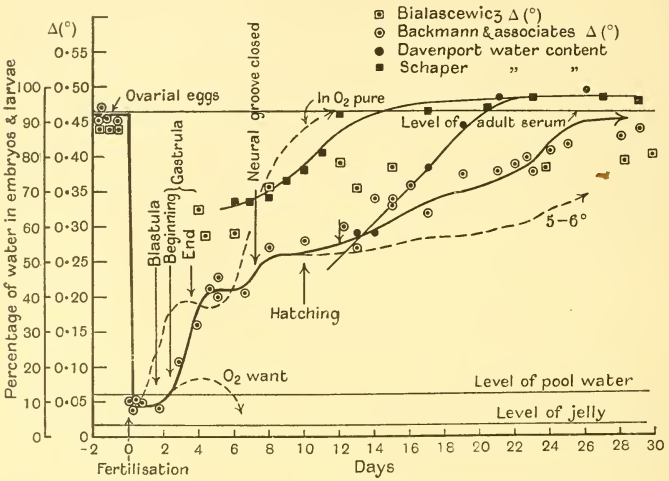


Fig. 177.

normally in solutions isotonic with adult serum or ovarial egg-contents, a strong indication that the behaviour of the osmotic pressure with time was a physiological phenomenon. This behaviour they studied at shorter intervals, and obtained the curves shown in Fig. 177. The isotonicity of the freshly fertilised egg was maintained, they found, during the formation of the blastula, and through gastrulation, but a rise occurred in the late period of the latter, continuing steadily for some time until the osmotic pressure reached about half the final value. Here there was a change in the rate of increase, which became much slower. Neither the closure of

the neural groove nor hatching affected it, and the rise continued without change until the osmotic pressure of adult serum was attained on or about the 25th day. These time relations held for *Rana temporaria*, but they were found to be applicable with variations to the eggs of many other amphibia. Backmann & Runnström found that parthenogenesis by hypertonic solutions induced at any rate the first of these changes, none of which, indeed, took place in unfertilised eggs. Micrometer measurements demonstrated that during the first few days exceedingly slight changes take place in egg-volume, quite insufficient in magnitude to account for the fall by simple dilution. E.g.:

| | % increase in diameter |
|---|---|
| 64-cell stage→blastula ... | 0·5 |
| Blastula→closure of blastopore | 0·4 |
| Closure→12 hours after closure | 4·9 |

Diameter of unfertilised egg 3·537 mm.; fertilised 3·79 mm.

During the later ascent of the osmotic pressure curve, there was a certain growth in volume, but Backmann & Runnström only gave a few fragmentary figures for this. The chorionic or perivitelline liquid seemed to be hypotonic to the 5-day old embryo, but hypertonic to the pool water, i.e. about $-0{\cdot}15°$. Backmann & Runnström regarded the change in the osmotic pressure of the egg as not of any recapitulatory significance, because some experiments which they made on land frogs gave the same results. Although the eggs normally developed on dry land, they showed the same osmotic pressure changes. They were more inclined to see in these effects an adaptation mechanism which had been retained by the land frogs although of no further use.

Backmann & Sundberg next confirmed de Varigny, who had stated in 1888 that solutions of different osmotic pressure were isotonic with frog's eggs at different stages of their development, without giving any experimental figures[1]. They also compared their gradually rising curve for osmotic pressure during larval life with the figures for water-content previously found by Davenport and Schaper. The result is shown in Fig. 178. There was undoubtedly a rise in water-content, which came to a level plateau at about 94 per cent. by the

[1] It must be remembered throughout this section that experiments of the type of de Varigny's, unlike freezing-point measurements, only give the osmotic pressure of the substances within the egg to which the membrane is impermeable.

20th day at the latest, and this rise was accompanied by the rise in osmotic pressure. Backmann & Sundberg regarded this finding as very significant, revealing as it did something of the mechanism by which the water-content of the embryonic cells was raised in the later stages. The entry of the water was, they thought, the factor which assured the maintenance of osmotic pressure within reasonable limits, as the saline ions, and perhaps other crystalloids, passed into solution

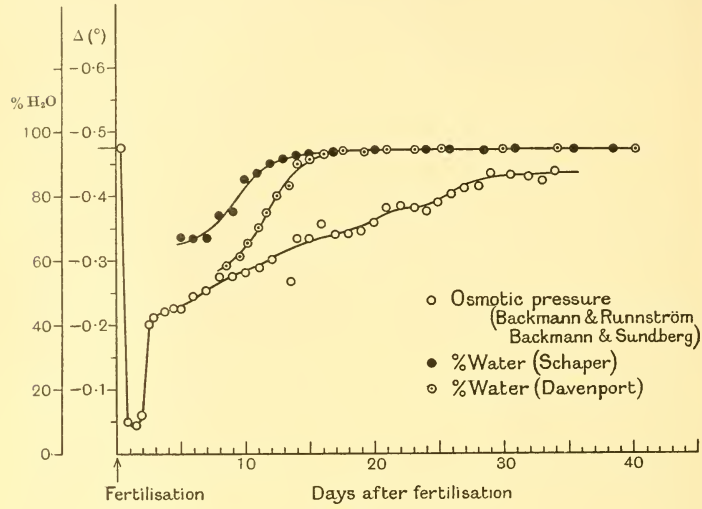


Fig. 178.

from the adsorbed condition. Backmann & Sundberg found that if unfertilised eggs of *Rana temporaria* were placed in distilled water there was no sudden fall of osmotic pressure as in the fertilised eggs, but a gradual swelling due to water-absorption and a decrease of Δ from − 0·48° to − 0·35° in 3 hours, i.e. to the stage reached normally by embryos by about the 20th day of development. This showed that, whatever the explanation of the normal behaviour might be, it was not due to simple absorption of water. From these premises it was to be expected that organs or tissues of frog embryos would react differently to salt solutions, if taken from different developmental

stages, and this was found to be the case by Harrison, who observed that explants of frog spinal cord from 4–5-day embryos would not grow in 0·7 per cent. salt solution (isotonic with adult serum), but would do so perfectly well in 0·4 per cent. Backmann & Sundberg pointed out that this corresponded with a $\Delta$ of $-0{\cdot}245°$, and so could be explained perfectly on the basis of Fig. 178.

The work was continued by Backmann, who measured with a micrometer the swelling of frog's eggs in different solutions. Previously some observations on this point had been made by Wilson and by Tonkov, but they were more concerned with the morphological changes caused by salt solutions. This technique afforded a means of checking the measurements already made of the osmotic pressure of the egg-contents, only now Backmann worked with the eggs of *Bufo vulgaris* and *Triton cristatus*. In both cases the unfertilised eggs remained without change of diameter for many hours in solutions of $\Delta - 0{\cdot}44°$, but the fertilised eggs (morulae) remained without change of diameter in solutions of $\Delta - 0{\cdot}02°$. The former eggs placed in the latter solution swelled up by 2·5 per cent. in 48 hours, the latter eggs placed in the former solution shrank by 1·2 per cent. in 24 hours. There was thus every indication that the results previously obtained on the frog held also for the toad and the newt. In these cases also, unfertilised eggs would have a $\Delta$ of $-0{\cdot}45°$ and the fertilised ones $-0{\cdot}02°$. The only difference between the frog on the one hand and the toad and the newt on the other was that, in the former case, there was no perceptible increase of volume after fertilisation, and, in the two latter cases, there was a slight normal increase.

In 1900 Bataillon had studied the conditions under which the eggs of the lamprey, *Petromyzon planeri*, would develop. He found that the fertilised eggs of this cyclostome would not develop properly in salt solutions above 0·2 per cent. In 0·5 per cent. the process would not go further than the gastrula stage, in 0·6 per cent. only as far as the morula, while in 0·8 per cent. no further than the 16-cell stage. From these data Backmann calculated that the osmotic pressure of the egg-interior in the morula stage of *Petromyzon* must be about $\Delta - 0{\cdot}125°$. No figures are available for the $\Delta$ of the adult serum of *Petromyzon planeri*, but Dekhuysen got a value of $-0{\cdot}49°$ for *Petromyzon fluviatilis*, from which it is probably legitimate to conclude that events proceed in the cyclostome egg just as in that of amphibia. The only fact still wanting is the osmotic pressure of

unfertilised ovarial *Petromyzon* eggs. Bataillon's data on *Ascaris* eggs might be treated in a similar way.

Later, Backmann, Sundberg & Jansson studied the effect of excess and lack of oxygen on the osmotic pressure curve during embryonic and larval life in the frog. Their results (which were never reported in full) are shown on Fig. 177. Development in almost pure oxygen led to a precocious augmentation of the osmotic pressure rise. They did not state, however, whether development in almost pure oxygen led to any shortening of the hatching time, or to more rapid differentiation or growth. Lack of oxygen was produced by causing the eggs to develop in water covered by a thick layer of paraffin oil. In such circumstances, the osmotic pressure of the eggs followed the usual course, until at about the 5th day the rise began to cease, and the gastrulae died shortly afterwards with an osmotic pressure of from $\Delta - 0{\cdot}09°$ to $\Delta - 0{\cdot}055°$[1]. The membranes seemed to lose their elasticity from the 3rd day onwards, and there was great swelling. Unfertilised eggs placed under similar conditions cytolysed after only 24 hours, their osmotic pressure having fallen to isotonicity with the pond water. Backmann went on to study the effects of temperature. Kept at 30 to 40° the eggs swelled a good deal, but in spite of that they had an osmotic pressure by the 3rd day which was up to normal, or a little above it ($\Delta - 0{\cdot}048$ at 40° and $\Delta - 0{\cdot}040$ at 30°). Kept at 5 to 6° the development was much retarded, and the rise in osmotic pressure, though at first behaving quite normally, was in its second phase much drawn out. Thus on the 21st day the eggs at the low temperature would have a $\Delta$ of $- 0{\cdot}30°$, while eggs at normal temperature (17°) would have $- 0{\cdot}39°$, and eggs at normal temperature but of the same morphological stage as the cold ones would have $- 0{\cdot}25°$. Backmann concluded that all these experiments showed a close association between morphological development and osmotic pressure, the latter entity being unable to vary more than a little independently of the former. As the temperature coefficient for osmotic pressure is exceedingly small, that entity must be dependent in its turn on some process with a marked temperature coefficient, i.e. whatever reaction or reactions were controlling the growth-process as a whole (see also on this p. 910).

[1] Thus lack of oxygen abolishes the mechanism which maintains the osmotic pressure difference, but the parallel with the vitelline membrane of the hen's egg is not close for here the embryonic development is affected too (see p. 817).

Thunberg criticised the statement of Backmann & Runnström that frog's eggs would not develop in solutions isotonic with the adult blood serum, and cited some not very good work of Overton's in support of the opposite view. But the observations of Backmann and his associates were later confirmed by Bialascewicz, who found the fall of osmotic pressure on fertilisation, the subsequent rise in osmotic pressure and the corresponding rise in water-content. Citing the experiments of Siedlecki, who had found the embryos of the Javanese frog (*Polypedatus reinwardtii*) incapable of developing in normal pond water if removed a day or two early from their envelopes, Bialascewicz concluded that the perivitelline liquid was not by any means ordinary water, but contained osmotically active substances which could not diffuse out through the egg-membrane, and so maintained a definite pressure gradient involving constant tension on the envelopes. The values which Bialascewicz obtained for osmotic pressure are shown in Fig. 179. He also made osmotic pressure determinations on ovarial eggs of other anura (*Rana esculenta* – 0·446°, *Bombinator igneus* – 0·445°). For ovarial eggs his Δ was – 0·444°, and for adult serum – 0·479°, a difference which he emphasised as resembling the corresponding difference in avian eggs. He explained the initial fall of osmotic pressure in the egg itself rather by an excretion of osmotically active substances into the newly formed perivitelline space than by an adsorption on to colloidal particles. Thus the perivitelline fluid would be a salt solution of definite strength, and he had himself previously shown that the embryos of *Salamandra* and *Molge* would

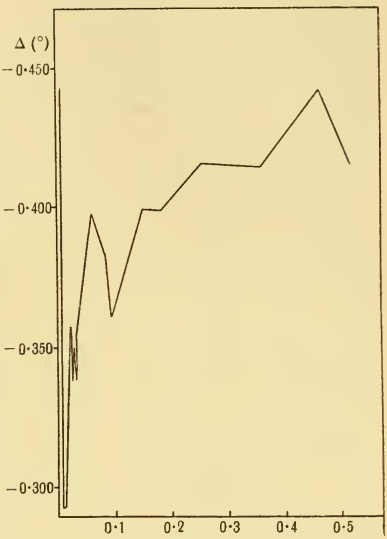

Fig. 179.

develop only in Ringer-Locke solution if removed from their envelopes before hatching, and not at all in pond water. With regard to the later rise in osmotic pressure, Bialascewicz pointed out that the adsorption explanation of Backmann & Runnström could not be right in view of the fact that the last traces of the yolk disappear some time before the osmotic pressure has reached its final level. On other grounds, moreover, Bialascewicz rejected any association between osmotic pressure and water-content, and certainly the curves in Fig. 178 do not go very well together. He preferred to postulate an increase in osmotically active substances derived from the food, and an important regulatory action on the part of the kidneys, organs now (in the later stages) quite functional in selectively retaining or excreting crystalloids.

The work of Backmann and his collaborators, and of Bialascewicz, was again confirmed by Przylecki, who paid special attention to the rôle of the perivitelline fluid. In contradiction with Backmann, however, he found that fertilisation was not the governing factor in the lowering of internal osmotic pressure in the eggs of *Rana temporaria* and of *Triton cristatus*, but that this occurred whether fertilisation took place or not. Absorption of water is not responsible, but rather the excretion of salts and water to form the perivitelline fluid. Przylecki (working on *Triton taeniatus*) agreed with Bialascewicz in not getting such a high freezing-point as Backmann for the just-fertilised eggs, i.e. $-0{\cdot}20°$ instead of $-0{\cdot}045°$. In a second paper, Przylecki studied the conditions necessary for the formation of the perivitelline space in the unfertilised frog's egg. The chief of these was the hypotonicity of the surrounding medium, a difference in freezing-point of only a few hundredths of a degree, however, being sufficient to set the process in motion. The trigger mechanism is probably the entry of a small amount of external water into the egg. The egg has to remain at least 30 minutes in the hypotonic medium, but after from 5 to 7 hours in a wet chamber the eggs, placed in water, can still develop perivitelline spaces. Electrolyte solutions of all kinds hinder the formation of the perivitelline space, from $\Delta - 0{\cdot}1°$ or $0{\cdot}08°$ onwards. Oxygen seemed to be necessary, for eggs in hydrogen and water would not produce the space, although all other conditions were favourable. Augmentation of $10°$ doubles the speed with which the perivitelline space is produced.

Work on the frog's egg was continued by Voss in 1926. By micrometric measurements he found that the size of the perivitelline space

was directly dependent on the osmotic pressure gradient between the inside and the outside of the egg. In distilled water it was greatest, in 0·004 per cent. sodium chloride less, and so on. Natural isotonicity was with 0·05 per cent. sodium chloride; the space was then normal in size. Over a considerable range it acted as an accurate biological osmometer, but ceased to do so when the concentration of salts outside became very high. During development the permeability of the egg-membranes varied; shortly after fertilisation, salts were let out, but not in the later stages. The more eggs developed in a given volume of water, the more ions diffused out in the early stages, and the smaller the perivitelline spaces were. As for the egg-jelly, it consists of two concentric portions, both of which are very sensitive to the concentration of ions in the external water, as may be judged by their rapidity of swelling after the eggs are laid (see p. 323). Hykeš has since found that in ordinary water frog's eggs develop quicker when freed from their jellies than they do inside them. In distilled water, however, they will not develop except within the jellies.

Voss's work agreed very well in general with that of McClendon. McClendon parthenogenetically fertilised the eggs of the leopard frog, *Rana pipiens*, by electrical stimulation in distilled water, and then estimated the salt content of the water after a definite interval. The results were as follows:

| | Chlorine (as c.c. 1/100 $N$) in water after 7 hours | | Total ash in mgm. after 7 hours | |
|---|---|---|---|---|
| Unfertilised ... | 1·2 | | 6·6 | |
| Fertilised ... | 2·05 | 2·01 | 9·6 | 10·3 |
| Stimulated ... | 1·98 | | 11·0 | |

The ash apart from the chloride contained sodium, lithium, potassium, calcium, magnesium, sulphate and carbonate, but no phosphates. There was no doubt that the diffusion of salts from the fertilised eggs (whether naturally or artificially) was nearly double that from the unfertilised eggs. McClendon had previously shown that in the case of sea-urchin's eggs a great many parthenogenetic agents caused an increase in permeability of the egg-membranes, and he regarded this as a general phenomenon. He thought that fertilisation, by allowing increased permeability, permitted the eggs to live, while if they were not fertilised they would swell up and die. Experiment showed that the mean diameter of unfertilised eggs placed in water for 30 minutes was 1·52 mm., while the mean diameter of

fertilised ones was only 1·47 mm. These relations confirmed Backmann's results; Backmann, indeed, had found that the unfertilised salamander egg burst in 2 to 7 hours, if placed in water, but not if placed in salt solution. McClendon observed that, if the fertilised eggs were placed in salt solutions, development often ceased at the gastrula stage, and he concluded that the exit of salts from the egg was a physiological necessity.

Bialascewicz's views on the importance of the perivitelline fluid were accepted by McClendon, who analysed it in the cases of *Amblystoma* and *Cryptorhyncus*, and found salts and organic substances, but only traces of protein (0·16 per cent. dissolved solids). Bialascewicz's finding, that immediately after fertilisation there was a measurable decrease in the diameter of the egg-cell, evidently reflected the formation of the perivitelline fluid. Backmann & Runnström had suggested that the fluid might be secreted by the suckers of the embryo, but the case of the salamander, which has no suckers, is contrary to this view. The normal course of events in the amphibian egg, therefore, after laying, is as follows: if fertilisation occurs, the osmotic pressure of the egg drops sharply to a low value, perhaps because of the intracellular fixation of osmotically active substances, but more probably because some of these are excreted into the perivitelline fluid, and to a certain extent into the surrounding medium. Then there occurs a gradual rise, leading back to the initial value, probably brought about by the increase of osmotically active substances produced in metabolism, and not by any absorption of salts from without. The skin of the adult frog absorbs water at a rapid rate, and if it were not for the action of the kidney the animal would die of oedema. The pronephros develops and is believed to be functional well before hatching, so the frog embryo and larva is well protected against this possibility.

## 5·2. The Genesis of Volume-regulation

More recently the problem of the origin of water regulation in amphibia has been investigated by Adolph, who studied the change in volume or weight when different developmental stages of *Rana pipiens* were placed in various solutions. The unfertilised eggs sometimes swelled, and sometimes shrank, but in none of the experiments did the change in volume follow exactly the concentration of the environmental salt solution. This was in contradiction with Back-

mann's results, but, although his were regular, they never exceeded plus or minus 5 per cent., while Adolph's were of the order of 30 per cent. As volume decreased in some dilute salt solutions, Adolph concluded that the egg-membranes were not completely permeable to sodium chloride. The next stage investigated was the yolk-plug stage. "The results were unexpectedly irregular and allowed of no

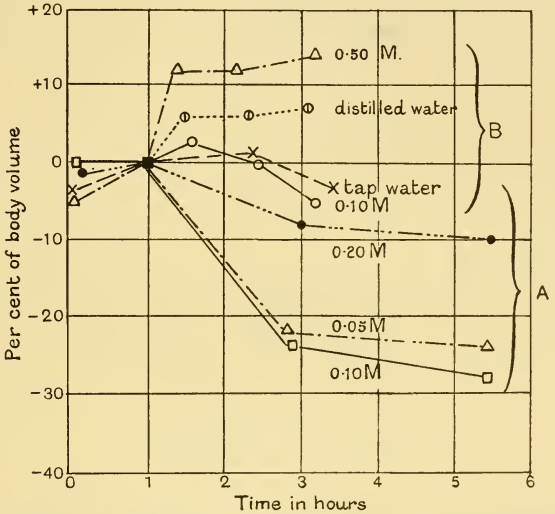


Fig. 180. Unfertilised eggs. A, Loss in volume; B, gain in volume.

precise definition of the relation between volume and concentration. Possibly the egg is comparatively semipermeable in the dilute solutions, but in stronger ones is made completely permeable to solutes." This would explain the relationships seen in Figs. 180, 181 and 182, taken from Adolph's paper. After hatching, the response became more regular, and predictions of the results could be made. Adolph concluded that from the unfertilised egg up to metamorphosis no marked changes in regulatory activity occurred in *Rana pipiens*. The only definite difference was that the eggs and embryos lost relatively less in volume when immersed in strong

salt solutions (such as 0·20 *M*), and the hatched larvae lost relatively more.

Adolph also made a very interesting observation in the later stages. Many observers had found that adult frogs gained weight in dilute salt solutions, but that tadpoles lost weight in solutions of the same concentration. Adolph found that there was a change over as regards this property about $1\frac{1}{2}$ days after the appearance of the fore limbs, and, in his opinion, it was quite sharp. At exactly the same time well-marked morphological changes occurred in the skin.

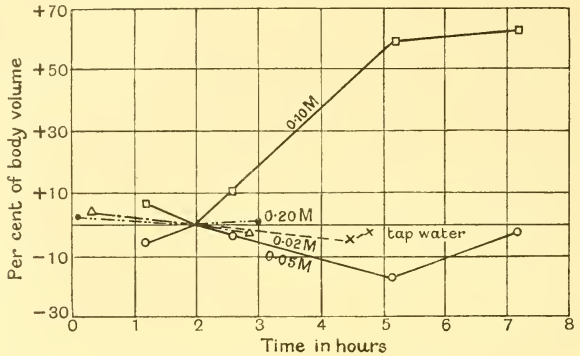


Fig. 181. Embryos at the yolk-plug stage.

"Extrusion of eggs from the body", says Adolph, "is followed by a gradual increase of volume by entrance of water from the fresh-water medium. Fertilisation changes all this and enables electrolytes to get out of the egg so that now concentration is sacrificed while volume is kept constant. Before hatching, the ability of the embryo to hold its electrolytes is regained and the ability to absorb solutes from a medium very dilute in them is acquired (Krizenecki). During this period the ability to regulate volume is sacrificed slightly, but with gain in mass it becomes possible for the larva to hold the volume as well as the concentration, and the losses are exactly compensated for on return to water. Soon after hatching, the tadpole is very independent of its medium with respect to both concentration and volume. At metamorphosis a change occurs in the mode of regulating in

various solutions. This is the ontogenetic history of the water relations of tadpoles." Adolph pointed out, with reference to the increasing water regulation apparent from the results of Backmann and his collaborators, that the most important factor was probably the embryonic ectoderm, for there is no evidence that the egg-membranes

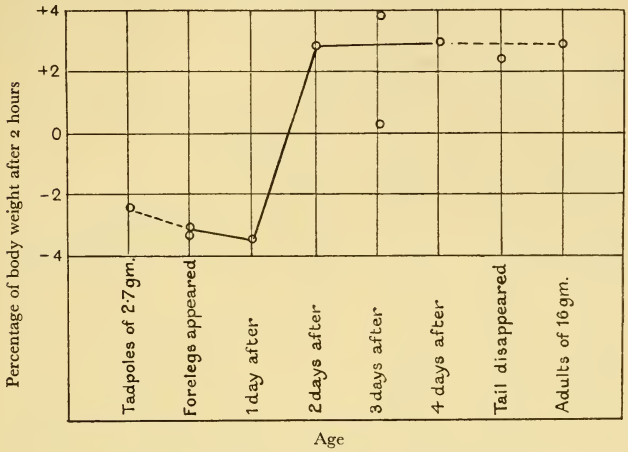


Fig. 182. The development of the power of regulating body-volume in *Rana pipiens*.

play any part in regulating the distribution of water between the body of the embryo and the environment of the egg.

Belehradek & Huxley subsequently found that the water-regulating power of Amblystoma; imperfect during the larval state, assumes its adult efficiency during metamorphosis. Konopacki's conclusions are also in general agreement with those of Adolph. He studied the effects of distilled water on amphibian eggs and embryos, and showed the coming-into-being of a regulatory mechanism, probably resident in the embryonic ectoderm. Konopacki also confirmed many of Bialascewicz's results with reference to the function of the perivitelline liquid in the frog's egg.

## 5·3. The Osmotic Pressure of Aquatic Arthropod Eggs

The osmotic behaviour of the amphibian egg is to some extent paralleled by that of the eggs of cladocerans, on which Przylecki has made notable studies. He found that the youngest embryos in his series had the lowest osmotic pressures; thus at 6 hours it was $\Delta - 0{\cdot}245°$ for *Simocephalus vetulus*, and $- 0{\cdot}186°$ for *Daphnia magna*. As development proceeded, the pressure steadily rose, reaching at 54 hours $- 0{\cdot}752°$ in the former case, and at 84 hours $- 0{\cdot}739°$ in the latter. The curve is shown in Fig. 183 taken from Przylecki's paper. The eggs were parthenogenetically "fertilised," and the osmotic pressure was not determined by direct freezing-point measurements, but by observing how strong a glucose solution was required to make no change in egg- or embryo-volume[1]. Przylecki regarded the rise in osmotic pressure as largely due to the formation of osmotically active substances in metabolism. At the 6th hour the egg-membrane is in a state of tension, which augments until the 24th hour (120 per cent.), but then ceases altogether up to the 60th hour, after which it rises again (to 167 per cent.), as is shown in Fig. 184. In spite of the mounting osmotic pressure, then, there is a period during which no increase in membrane tension takes place[2]. At this time the membrane does not return to its original state if the pressure is relieved by pricking the egg, but has evidently expanded in a more plastic manner. At the 60th hour the outer membrane (egg-membrane) bursts, and the larval membrane begins to expand. The growth of the embryo in volume follows these limiting factors.

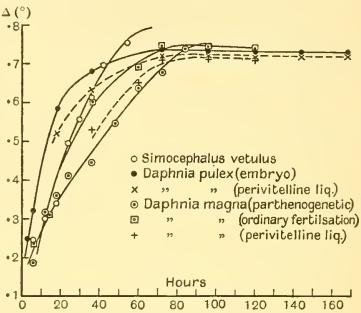

Fig. 183.

In a second paper, Przylecki confirmed the earlier results, working

[1] And assuming that the permeability of the egg-membranes for glucose was the same as that for salts.

[2] Strictly speaking, this is not tension, but rather the amount by which the elastic limit is exceeded.

with fertilised eggs of *Daphnia pulex* and *Daphnia magna*. All his results are shown in Fig. 183, from which it will be seen that the osmotic pressure of the perivitelline liquid rises *pari passu* with that of the embryo, but at a rather lower level.

The cladoceran embryo, like that of the silkworm, passes through a hibernation period when part of its development has been completed. Przylecki found that during this time the osmotic pressure remained at the final level reached after the main rise, i.e. from $-0{\cdot}734°$ to $-0{\cdot}74°$, but after two months there was a lowering of 30 per cent. or so, followed by a rise until hatching. Przylecki estimated that of this lowering about 24 per cent. was caused by absorption of water, and 34 per cent. by excretion of osmotically active substances into the perivitelline fluid, as in the early stages of the frog's egg, while the remainder he could not account for. Przylecki gave no figures for the osmotic pressure of the unfertilised *Daphnia* egg, but presumably it was somewhere about $-0{\cdot}7°$, in which case the events in amphibians are closely paralleled by those in cladocerans (see Fig. 185).

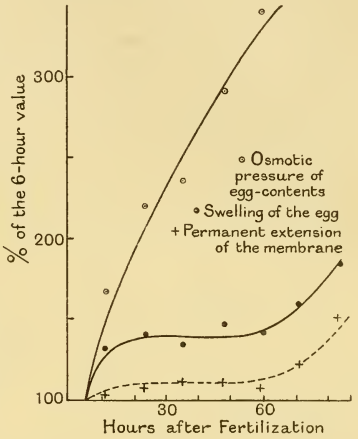

Fig. 184.

Osmotic pressure seems to play a very important part in the normal embryonic life of cladocerans. Ramult, working on *Daphnia pulex*, found that if these animals, soon after the eggs had been laid in the brood-pouch, were transferred from pond water to sodium chloride solutions the embryos did not hatch at the proper time. During normal embryonic life, two membranes burst, first the egg-membrane, and then the larval membrane. Although differentiation goes on continuously, growth does not, but has periods of slowness while waiting for the membranes to burst, the larval one being more extensible than that of the egg. In "closed development", artificially induced by a rise in osmotic pressure of the environment,

differentiation proceeds normally in spite of the suppressed growth, so that well-formed dwarf embryos are produced. These will never hatch if the osmotic pressure of the exterior remains high, and will die within the membranes. The necessary minimum osmotic pressure for "closed development" is that of $N/30$ NaCl. Embryos artificially liberated from their membranes will continue to develop normally in either pond water or salt solution of the same strength as that which prevents hatching, or even in stronger salt solutions. The volume of the finished embryo in the cladocerans is at least three times as great as that of the egg before the bursting of the egg-membrane, and as this increase in volume can be completely inhibited by raising the osmotic pressure of the external liquid, there can be no doubt that the cladoceran egg is arranged to absorb a great deal of water from its environment. This property is of much interest and will be referred to again in the succeeding section (see p. 896). In distilled water the preponderance of the inner over the outer pressure at hatching would be about $\Delta - 0{\cdot}75°$ but in pond water a little less, as the latter has itself an osmotic pressure of $\Delta - 0{\cdot}02°$. At external osmotic pressures of about $\Delta - 0{\cdot}176°$, 50 per cent. of the eggs go into "closed development", which shows that half of them can, if necessary, raise their internal osmotic pressure to higher than this. But they cannot go further, and though they may overcome $N/20$ NaCl they will not manage $N/15$ NaCl. Ramult's discovery of "osmotic hatching" in cladoceran eggs is paralleled by the fact that phyllopod eggs (e.g. *Artemia salina*) will not hatch in the strong salt solutions in which the adults normally live (Becking). This is a remarkable instance of an experiment done for us by Nature.

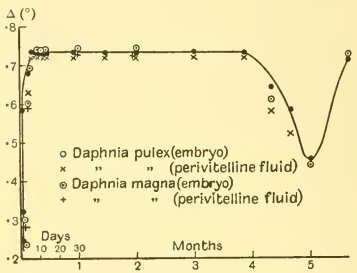

Fig. 185.

The eggs of other arthropods have been little investigated from this point of view. Walther in 1913 placed the eggs of the crab *Telphusa fluviatilis* in water to which magnesium salts were added, and found by microchemical methods that not until many days had elapsed was any magnesium to be found inside the egg-membranes. In other eggs, of course, the salt penetrates much more quickly,

e.g. Stockard's magnesium (cyclopean) embryos. Roffo & Correa studied the osmotic properties of the egg-membrane of the mollusc *Voluta brasiliense*, and found that they showed no changes during the development of the embryo.

## 5·4. The Osmotic Pressure of Fish Eggs

A different state of affairs came to light, however, when fish eggs were investigated. Runnström, for instance, examined the eggs of *Salmo* by counting the number which developed normally in different solutions. Nordgaard had previously found that these eggs would not develop in sea water, or in 20 per cent. salt solution, though, if fertilisation had been done in fresh water, they could stand 9 per cent. Runnström and Svetlov measured the depression of the freezing-point of the egg-contents (excluding the perivitelline liquid) of *Salmo savelinus* and *Salmo fario* respectively, obtaining the following results:

| | Δ (°) | Investigator |
|---|---|---|
| Oviduct eggs ... ... ... | −0·645 | Runnström |
| Eggs 4 hours after fertilisation ... | −0·599 | ,, |
| Hatched larvae ... ... ... | −0·580 | ,, |
| Blood of full-grown fish ... ... | −0·636 | ,, |
| Egg throughout development ... | −0·500 | Svetlov |
| Perivitelline liquid ... ... | −0·010 | ,, |

Evidently the changes were very small. The initial slight decrease can be accounted for, according to these workers, by an absorption of water from the hypotonic (fresh water) medium and an increase in the amount of perivitelline liquid, for after 4 hours in water there is a 6 per cent. increase in weight, just as, in the case of *Salmo trutta*, Miescher found an increase of weight of 10·83 per cent. in the same circumstances, and Bogucki an increase of 20 per cent. volume. In *Osmerus eperlanus*, again, there was no fall or rise of osmotic pressure. Thus the eggs of fresh-water teleosteans are practically independent of the hypotonic medium in which they live, and the same independence is shown by marine teleosteans with regard to their strongly hypertonic medium, as will be shown below. On the other hand, as Nordgaard's investigations showed, the fresh-water teleost egg cannot be said to be indifferent to hypertonic solutions. Development in 8·5 per cent. salt solution would be development in a practically isotonic solution, and, as we have seen, this is as much as the *Salmo*

egg will stand. Ramult has found, however, that *Salmo trutta* eggs are more resistant than other varieties, a fact probably connected with the adult habits of the fish which inhabits fresh and salt water indiscriminately.

The osmotic pressure of the contents of the teleost egg, therefore, is constant throughout development, and so differs profoundly from that of the amphibian egg. Runnström noticed that the membranes of oviduct eggs were very fragile and easily broken, whereas immediately after laying they were hard and tough. Comparative centrifugation and experiments on solubility in 3 per cent. potassium hydroxide easily demonstrate this. In 10 per cent. salt solution the hardening does not take place. Obviously these changes in the egg-membranes are of great importance with respect to the osmotic behaviour of the teleost egg, and perhaps may be regarded as the causes of its special properties. Runnström concluded that another function of the membrane was protection against polyspermy. He also made experiments with potassium chloride solutions, finding the time taken for the salt to diffuse through the membranes and stop the heart-beat. In this way he observed that the egg-membranes were much less permeable than the skin of the hatched larvae, just as Loeb had previously done for *Fundulus* embryos. For further details see the section on Susceptibility and Resistance.

In considering the difference between the behaviour of teleosts and amphibia as regards osmotic pressure, it must be remembered that during development up to hatching, and for a short time afterwards, the water-content of both amphibian and teleost embryos (or rather embryo plus yolk) is steadily increasing. Yet amphibian eggs will not develop properly in solutions isotonic with the adult tissues, while those of teleosts will.

Further details are afforded us through the researches of Bogucki, who set up the egg-membranes in microdialysers and studied the passage of substances through them. In this way he found that they were penetrable by chlorides, monosaccharides and amino-acids, but not by proteins, polysaccharides or colloids such as Congo red. During development the permeability was not modified: the same amount of potassium chloride dialysing through in a given time at 10 or 30 days from fertilisation. The initial intake of water after fertilisation was correlated by Bogucki with the formation of the perivitelline liquid, which just accounts for the change. Bogucki

found that this absorption of water was stopped by hypertonic electrolyte solutions, but not by hypertonic solutions of non-electrolytes. The ordinary laws of diffusion and osmosis, therefore, will not explain the formation of the perivitelline liquid. This conclusion is in accordance with the views of Svetlov. Hayes found in the case of the Atlantic salmon, *Salmo salar*, that the egg membranes are impermeable to electrolytes or colloidal substances.

Gray has also worked on the trout egg. He began by enquiring what the forces were which maintain the teleostean egg's high concentration of electrolytes against the low osmotic pressure of the fresh-water medium, and where they were located. The membranes could not, he thought, be impermeable to electrolytes, for the embryo could not then take in any inorganic substances from outside. Moore; Osborne; and Donnan had all urged that the presence of colloidal substances inside a cell would cause a differential distribution of inorganic ions within and without, but Gray was not convinced that this would explain the state of affairs in the trout egg.

Gray found experimentally that death of the eggs was accompanied by a marked increase of electrolytes in the surrounding water. Thus the resistance of the water in which eggs were standing was as follows:

| | Ohms |
|---|---|
| Before shaking ... ... | 3550 |
| 1 hour after shaking ... | 305 |

This was confirmed afterwards by Kronfeld & Scheminzki, who got less striking figures:

| | Ohms |
|---|---|
| Before addition of alcohol | 3200 |
| 3 hrs after addition of alcohol | 2700 |

But during normal development no appreciable amount of electrolytes was lost from the eggs; thus, in an experiment where the original resistance of the water was 6520 ohms, after 3 hours in contact with fertilised eggs it was 5000 ohms, a difference amply accounted for by carbon dioxide production. Direct measurement on egg-*Breis* before and after fertilisation showed that the amount of diffusible electrolyte within the egg is hardly affected at all by this event.

| | Ohms |
|---|---|
| Unfertilised ... | 1040 |
| Fertilised ... | 1070 |

Similar determinations done on eggs and embryos of different ages showed no change over 38 days, as seen in Fig. 186. Again, the osmotic pressure, cryoscopically determined, was the same before fertilisation as between the 3rd and the 10th days, i.e. $-0{\cdot}48°$, though this figure was rather lower than that found by Bottazzi for the adult blood of the same species (*Salmo fario*), namely, $-0{\cdot}567°$. In the case of *Fundulus*, also, Loeb & Wasteneys found no difference in the depression of the freezing-point of the egg-contents before and after fertilisation (in both cases $-0{\cdot}76°$). On the other hand Bogucki found the $\Delta$ of trout eggs *which had never been wetted*, to be $-0{\cdot}64°$ while at the 8–12 blastomere stage it was $-0{\cdot}42°$.

From all these facts, and those mentioned on p. 334 in the section on Constitution, the conclusion is indicated that the factor which retains in the normal egg sufficient electrolytes to keep the ovoglobulin or ichthulin in solution is located in the protoplasmic membrane, which, thickened at one part to form the germinal disc, extends right over the egg-surface underneath the thick external membrane. The crucial experiment to test this view as against the Moore-Osborne-Donnan theory was to dialyse the egg-contents in a parchment thimble. If the electrolytes were retained in the egg by chemical affinities alone, they should not pass out into the dialysate. But experiment showed that that was just what they did, giving the curves shown in Fig. 187. The protoplasmic egg-membrane, therefore, cannot be merely impermeable to colloids and permeable to electrolytes, but must possess a certain degree of impermeability to the latter.

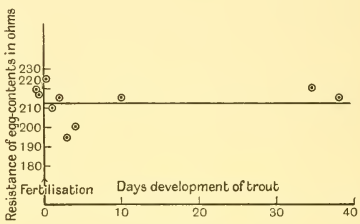

Fig. 186.

The actual process of elimination of electrolytes into the surrounding water by injured trout eggs was investigated by Gray in a subsequent paper, using Blackman's exosmosis apparatus. The electrical conductivity of the external medium was measured. The curve resulting was sigmoid, presumably because during the first period the cell-membrane was breaking down, and during the second period the electrolytes were diffusing away according to simple laws.

The independence of the environment which has been noticed in fresh-water teleosts extends also to marine teleosts, where there is a sharp contrast as against elasmobranchs. Dakin obtained the following values by direct freezing-point determinations:

| | Δ (°) |
|---|---|
| Sea water ... ... ... ... ... | −1·91 |
| Egg-contents of *Pleuronectes platessa* ... ... | −0·70 |
| Adult blood of *Pleuronectes platessa* ... ... | −0·75 |
| Egg-contents of *Scyllium canicula* ... ... | −1·80 |
| Adult blood of *Scyllium canicula* ... ... | −1·90 |

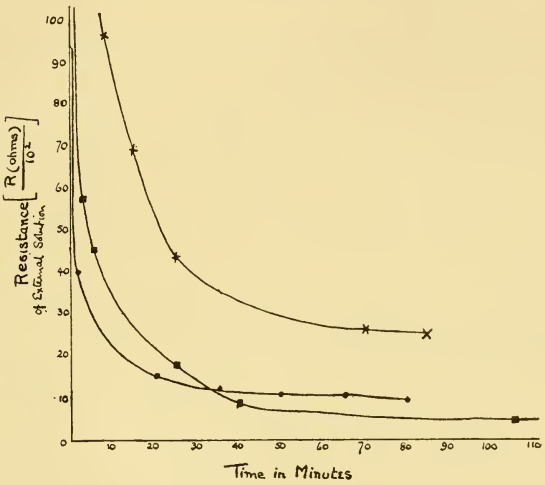


Fig. 187.

This was in agreement with Bottazzi's well-known findings that the osmotic pressure and salinity of teleostean blood was different from the sea (though it varied with it), while the osmotic pressure and salinity of elasmobranch blood was the same as the sea. Death of the plaice egg destroyed its osmotic independence. Thus the teleost egg, floating in sea water of salinity 35 per cent., had an osmotic pressure corresponding to a salinity of only 14 per cent.; correspondingly it will develop perfectly well in a mixture of 80 per cent. fresh and 20 per cent. salt water. With regard to *Scyllium* eggs, Dakin

found the horny cases to be quite permeable to salt, by simple osmometer measurements (see also p. 331 in the section on Constitution). Jacobsen & Johansen made similar observations, finding that the Δ and salinity of plaice eggs varied somewhat with the environment, but was always much lower than that of sea water. Death permits the entrance of salt, and the eggs sink to the bottom. Certain observations have also been made on the adaptation of fish eggs to different salinities; thus Amemiya found that the eggs of the anadromus "Ayu" fish, *Plecoglossus altivelis*, will not hatch in water of salinity above 20 per cent., 22 per cent. is very quickly lethal, and 15 per cent. optimum.

Osmotic pressure experiments with the eggs of *Fundulus* were done by Loeb & Cattell and by Loeb & Wasteneys. They studied the various antagonistic effects which electrolytes display on the stoppage of the heart-beat of the embryo. Embryos cannot recover from potassium chloride poisoning without the aid of other electrolytes, so Loeb & Cattell studied the efficiency of the different anions and cations. Loeb & Wasteneys concluded that the egg-membrane of *Fundulus* is almost impermeable to water and salt under normal conditions.

Fragmentary results on other fish eggs have been obtained by Ziegelmayer, who tested the effects of hormones and of various other substances on the size of *Leuciscus* eggs.

McClendon found, with Loeb & Wasteneys, that the egg-membranes of *Fundulus* were practically impermeable, and showed that, when by poisons this impermeability had been abolished, abnormalities occurred. Later, McClendon observed that the action of various toxic solutions markedly increased the permeability of the membranes to salts, but that their action was inhibited to some extent by anaesthetics. This last effect was confirmed in detail with the eggs of the pike, *Esox*. Anaesthetics that retarded development (2 to 3 per cent. alcohol or 0·5 per cent. ether) tended to inhibit the permeability-increasing action of a 1/10 molecular solution of sodium nitrate. Osterhout showed in 1914 that plant cells were made more permeable with increase of temperature, and McClendon extended this finding to the eggs of *Esox*, which is interesting in view of Ephrussi's results on echinoderm eggs (see p. 806). Loeb used the egg-membranes of *Fundulus* in many experiments on ionic permeability, e.g. specific gravity tests

In 1928 Sumwalt pointed out that the *Fundulus* embryo was enclosed in two membranes, in the early stages, by the outer chorion or egg-membrane and the vitelline membrane, and, in the later stages, by the chorion and the skin. The importance of the skin factor in the ontogenesis of water regulation, etc., had already been emphasised in the case of amphibia by Adolph, but Sumwalt used a new method, measuring the permeability to ions of the various embryonic membranes of *Fundulus* in terms of concentration potentials across the membranes between solutions of $N/10$ and $N/100$ potassium chloride after Michaelis. By means of a capillary pipette in a micro-manipulator, one electrode was introduced into the inside of the egg, and a similar electrode dipped into the solution in which the egg was placed. For a measurement of concentration potential through the chorion alone, the electrode was placed in the sub-chorionic or perivitelline space; for a measurement across the chorion plus the embryonic skin, it was put in the yolk-sac of the embryo. The results showed that the compound membrane of skin plus chorion could produce much greater concentration potentials than the chorion alone, the average for the latter being 19·4 millivolts, and for the former 55·2. In both cases change to the dilute solution (sea water diluted 100 times) caused the inside of the egg to become negative to the outside, indicating a relatively greater impermeability of the membranes to anions than to cations. This is less pronounced in the chorion than in the skin. Measurements of electrical resistance confirmed this view, for the resistance of the chorion alone was about 45,000 ohms, but that of the embryonic skin about 208,000.

## 5·5. Osmotic Pressure and Electrical Conductivity in Worm and Echinoderm Eggs

Of the osmotic pressure of oligochaete worm eggs very little is known, but there is in this connection an interesting study by Svetlov of the eggs of the Lumbricidae, *Bimastus constrictus*, and *Eisenia foetida*. The Terricolae, the sub-order to which the earthworms belong, lay their eggs in cocoons, which were formerly mistaken for the eggs themselves. These cocoons are brown and horny and vary in size according to the species; they contain ova and spermatozoa as well as a milky nutritive fluid in which the young worms float and by which they are nourished before they hatch out from the cocoons. Svetlov had morphological and cytological reasons for supposing

that in the early stages of *Bimastus* the three micromeres acted as osmoregulators for the rest of the embryo by excreting water, but that this was not so in *Eisenia*, and when he came to estimate the osmotic pressure of the milky liquids in the cocoons he found that the two were indeed different. The cocoon liquid of *Bimastus* gave a freezing-point depression of – 0·06° corresponding to 0·78 atmosphere or 0·108 per cent. sodium chloride, while that of *Eisenia* gave one of – 0·39° corresponding to 4·8 atmospheres or 0·68 per cent. sodium chloride. Svetlov found that the cocoon shells, although hard, were not impermeable, and showed, in fact, the properties of semipermeable membranes. Taking then samples of liquid from the normal habitat of the two worms, humus earth infusion for *Bimastus* and dunghill infusion for *Eisenia*, he found that the former was much less concentrated than the latter, the relation being the same as that found between the milky contents in the two cases. Svetlov constructed micro-osmometers with the cocoons from the two species and found that the permeability of *Bimastus* was not quite double that of *Eisenia*. Although the process was complicated in *Eisenia*, cocoons (of both species) placed in solutions of known osmotic pressure would come gradually into osmotic equilibrium with them. The osmotic pressure of the cell-interior of the eggs themselves was probably alike in both cases as the osmotic pressure of the adult blood was the same, and therefore, *Eisenia*, having, according to Svetlov's view, either abandoned or never evolved the osmoregulator micromeres of *Bimastus*, has to lay its eggs in the dungheap liquid or "Mistflüssigkeit" which has a high osmotic pressure.

The osmotic relations of the eggs of nematode worms have only once been investigated—by Szwejkovska. Between fertilisation and the first cleavage there occurs, she found, a great diminution in the size of the egg-cell while the egg as a whole remains unaltered in size. The egg swells only slightly in hypotonic solutions. By the plasmolysis method, she found the freezing-point depression of the egg-interior to be – 0·599° in the unfertilised egg, – 0·629° in the fertilised egg before the elimination of the first polar body, and – 0·636° after the elimination of the first polar body.

A great deal of work has been done on the permeability and osmotic pressure of echinoderm eggs. The jellies surrounding them when unfertilised, and which can be made visible by the Indian ink method, have been found to take up salt from the water. Glaser noticed

that the specific gravity of sea water in which *Arbacia punctulata* eggs had been standing for some time (1 hour) was always lower than that of ordinary sea water (1·0229–1·0219). This led him to ask whether the eggs or the jellies had abstracted any inorganic material from the water, and in fact experiments showed that there was always a deficit of from 2 to 7 tenths of a milligramme per cubic centimetre of chlorine in the egg-water after an hour when 1 part of eggs were suspended in from 7 to 10 parts of water. The absorption was real, for eggs which had taken up all the chlorine that they would in one vessel took up no more when transferred to another; therefore the phenomenon was not due to an interference with the silver nitrate titration on the part of any egg-secretion. Eggs from which the jellies had been removed did not show this behaviour, and, as histo-chemically the jelly was found to be rich in chlorine, everything pointed to the jelly being responsible (see Fig. 188).

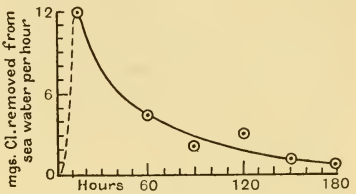

Fig. 188.

Most of what we know about the osmotic pressure of the egg-contents in echinoderms has to be deduced from what has been found to happen as regards the permeability of their membranes. McClendon in 1910 studied the electrical conductivity of masses of eggs before and after their fertilisation (*Toxopneustes variegatus* and *Tripneustes esculentus*) in a specially devised conductivity vessel. The following typical figures were obtained:

| | Conductivity | |
|---|---|---|
| Unfertilised ... | 0·01182 | 0·01153 |
| Fertilised ... ... | 0·01537 | 0·01277 |

There was undoubtedly an increase of electrical conductivity at the beginning of development. This might have been due (*a*) to increasing permeability of the egg-membranes, (*b*) diminution of fatty phase of the egg, (*c*) dissociation of protein-electrolyte complexes. The second of these possibilities was quickly ruled out by centrifugation experiments, which showed that just as much fat and oily matter was present after fertilisation as before. Cytolysis experiments showed that the first one was the most probable, and that as in

Höber's erythrocytes, the chief factor producing electrical resistance in eggs was the membrane. This conclusion was supported by various other workers, such as Lyon & Shackell, who reported that salts would enter and leave the fertilised *Arbacia* egg more easily than the unfertilised. Their only exception was iodine, which seemed to show the reverse behaviour. Harvey found that eggs became more permeable to sodium hydroxide after fertilisation, and Lyon & Shackell made similar observations with vital dyes. McClendon concluded that there was probably no liberation of electrolytes after fertilisation, but rather an increase of permeability to outside electrolytes. Parthenogenesis gave identical results with fertilisation.

McClendon's results were in general confirmed by Gray[1]. Invariably there was a decrease in electrical resistance immediately after fertilisation, as the following figures show:

| | Percentage decrease of electrical resistance on fertilisation |
|---|---|
| *Echinus acutus* ... ... ... | 11·2 |
| *Echinus miliaris* ... ... ... | 10·9 |
| *Echinus esculentus* ... ... ... | 12·5 |
| *Asterias glacialis* ... ... ... | 7·4 |
| *Strongylocentrotus lividus* ... ... | 36·0 |
| *Sphaerechinus granularis* ... ... | 23·0 |
| *Arbacia pustulosa* ... ... ... | 15·0 |

but half-an-hour or more after fertilisation this decrease was not so apparent. Gray concluded that the entrance of the spermatozoon into the egg causes an increase in electrical conductivity which attains its maximum within ten minutes, and is followed by a return to the value for the unfertilised egg. In later experiments he was unable to confirm this return to the original level, and published curves showing a gradual fall in resistance. Parthenogenesis, he found, did not give quite the same effect as normal fertilisation; thus by different methods, *Sphaerechinus* eggs gave increases of conductivity of 21·7, 6·8, and 6·0 per cent., instead of the normal 23 per cent. Hypertonic sea water markedly increased the conductivity of normal unfertilised eggs, the increase actually taking place while the eggs are in the hypertonic solution, but after artificial membrane formation, the conductivity was unaltered by this treatment. Change in the internal *p*H of the egg-cells, as found by exposing *Arbacia* eggs to ammonia solutions and noting the colour change of the pigment inside, produced no alteration in electrical conductivity. To support

[1] But see the criticisms of Cole regarding this (p. 829).

further his view that the properties of the membrane were the controlling factors in egg permeability, Gray put a list of haemolytic agents next to a list of parthenogenetic agents, and emphasised their general resemblance; Dalcq has followed this relation further in his book on fertilisation, which should be consulted for further details.

The work of Gray and McClendon on the conductivity of echinoderm eggs was extended by Bataillon to the eggs of amphibia. Bataillon found in their case also a regular decline in electrical resistance after fertilisation, but his results differed from the final ones of Gray, in that with the frogs there was always a gradual return to a level slightly less than that of the unfertilised eggs. The percentage decreases were as follows:

| | Percentage decrease of electrical resistance on fertilisation |
|---|---|
| *Rana fusca* (parthenogenesis) ... ... | 10·20 |
| *Rana esculenta* (fertilisation) ... ... | 12·35 |
| *Bufo calamita* (fertilisation) ... ... | 19·65 |

which agree well with those found by Gray. Bataillon's data are plotted in Fig. 189. He reported that the hepatopancreatic juice of the crab would destroy unfertilised amphibian eggs, but not fertilised developing ones (jelly having been removed in both cases), yet this immunity did not appear until after 30 minutes from fertilisation. He thought that the decreasing membrane permeability as measured in terms of electrical resistance might be connected with this fact.

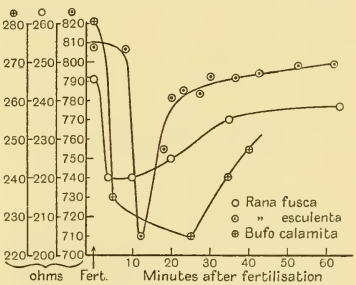


Fig. 189.

Other investigations of permeability of marine egg-membranes were those of McCutcheon & Lucke, who found that in solutions of hydrochloric acid, sodium hydroxide, carbon dioxide and ammonia no swelling of *Arbacia* eggs occurred, and they decided that the membrane was normally almost impermeable to these substances. Lillie made similar experiments. Driesch and Konopacki studied the cytological effects of raising echinoderm eggs in hypotonic sea-water. Fauré-

Fremiet investigated in some detail the osmotic relations of *Sabellaria* eggs. Placing them in solutions of glucose of $\Delta$ varying from $-0{\cdot}566°$ to $-0{\cdot}686°$, he measured their volume with a micrometer scale and found, as was expected, a regular change; thus at $-1{\cdot}135°$ the calculated volume was $21{\cdot}9 \cdot 10^{-8}$ c.c., and at $-3{\cdot}73°$ it was $9{\cdot}95 \cdot 10^{-8}$ c.c. Assuming that the membrane is impermeable to

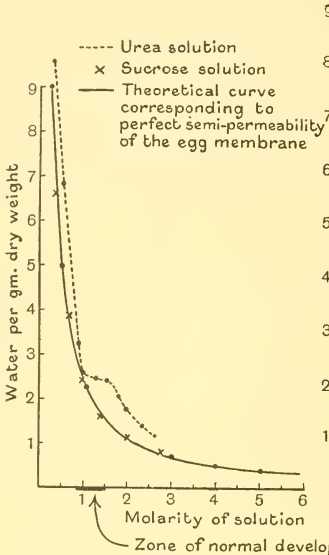


Fig. 190.

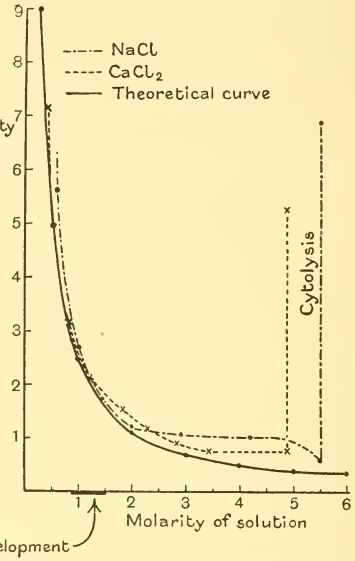


Fig. 191.

anything except water, Fauré-Fremiet calculated the imbibition at the different osmotic pressures. The normal *Sabellaria* egg contains 2·25 gm. of water for each gm. of solid, therefore a table was constructed showing the amount of water experimentally found in the eggs in the different sugar solutions, and the amount of water which should theoretically have been there. The observed and calculated values corresponded very well, as can be seen in Fig. 190, from which Fauré-Fremiet concluded that it would be right to assume that the egg-membranes were permeable only to water. The

difference between the theoretical and calculated figures were of the same sign, i.e. the eggs always contained slightly less water than on the complete impermeability hypothesis they should have done. Fauré-Fremiet explained this by suggesting that a very small degree of permeability to electrolytes was present. Data for urea solutions gave the same results, and are shown plotted in the same figure. Fig. 191 shows similar experiments done with neutral salts, such as sodium chloride, magnesium chloride and calcium chloride. Here, however, at the higher osmotic pressures the calculated and observed values diverge to a significant extent, which indicates that the absolute impermeability of the membrane is not maintained under such conditions. Probably electrolytes then enter the cell, just as in sugar solutions they may to a slight extent come out of it. These effects were so slight, however, that Fauré-Fremiet was inclined to see in them an adsorption of ions on the external surfaces of the egg-membrane rather than a true permeability. Acids and bases did not seem to penetrate at all into the eggs of *Sabellaria*. Temperature had a slight effect on the imbibition of water by the eggs; outside a constant range between 18° and 25°, the imbibition increased at low and decreased at higher temperatures. The margins of temperature between which the imbibition is at its normal value corresponded exactly to those between which perfectly normal development is possible. These effects have obviously an important bearing on the problem of egg viscosity, which has been handled by so many workers. Again, there was a modification of imbibition by the eggs according to change in *p*H—at *p*H 5 they contained slightly less water than normal, at *p*H 7, 6 per cent. more (*p*H 8·4 normal), and at *p*H 12, 10 per cent. more. These small differences were perhaps related, according to Fauré-Fremiet, to the isoelectric point of the egg-proteins. The effects of these various agents on the imbibition, the water-content, and therefore the osmotic pressure of the egg-contents, were of the following comparative magnitudes:

| | Maximum % variation |
|---|---|
| External osmotic pressure ... ... | 180 |
| Specific action of cations ... ... | 70 |
| Temperature ... ... ... ... | 14 |
| *p*H ... ... ... ... ... | 7 |

The heat factor was subsequently examined in more detail by Ephrussi, using the eggs of sea-urchins. He obtained an exactly

similar curve to Fauré-Fremiet's for the effect of heat on imbibition of water, and in collaboration with Neukomm for the effect of *p*H on imbibition (see Figs. 192 and 193). McCutcheon & Lucke, on the other hand, maintained that for *Arbacia p*H had no effect, but their measurements were rather few. The spermatozoa were, Ephrussi found, less susceptible to heat than the unfertilised eggs. The normal osmotic pressure of the egg-contents in *Strongylocentrotus lividus* was 25 atmospheres, i.e. practically isotonic with sea water. Heating at

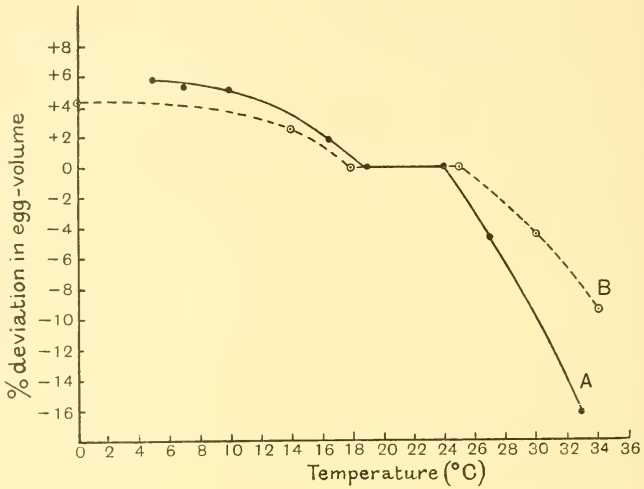


Fig. 192. A. *Strongylocentrotus*. B. *Sabellaria*.

different temperatures for a short time led to (*a*) a rise in the internal osmotic pressure, and (*b*) a subsequent fall, the whole curve forming a peak, the angle of which was the more acute the higher the temperature. Ephrussi concluded that two irreversible processes were in operation.

Perhaps the most interesting result obtained by Fauré-Fremiet on *Sabellaria* eggs in this connection was the finding that the eggs obeyed the Boyle-Mariotte law. Ephrussi & Neukomm could not show that this was so for *Strongylocentrotus* eggs. If $pV = K$ holds good, the eggs should swell in hypotonic solutions and shrink in hypertonic

solutions to an extent just sufficient to keep the product of the equation constant, but the sea-urchin's eggs did not do this. Fig. 194 shows on curve *A* the actual behaviour of the eggs, volume being plotted against osmotic pressure of external medium, and *B* the theoretical curve which should have resulted if the Boyle-Mariotte law had been followed. It is evident that the divergence is greatest in very hypotonic solutions, nil in normal sea water, and again

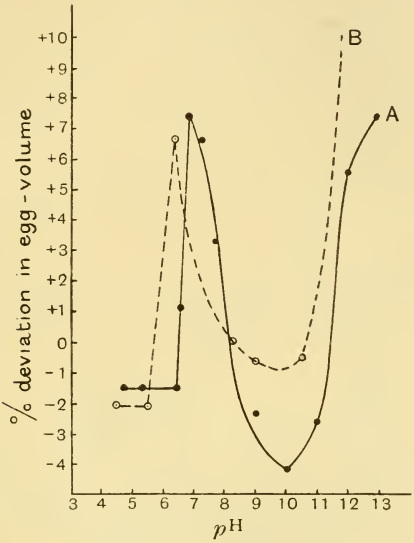


Fig. 193. A. *Strongylocentrotus*. B. *Sabellaria*.

marked in hypertonic solutions. In other words, the sea-urchin's egg opposes a certain amount of resistance to extreme hydration on the one hand, and to extreme dehydration on the other. Ephrussi & Neukomm offered no explanation for these facts, but thought it as difficult to picture any considerable amount of glucose getting into the egg from hypertonic solutions as to picture any electrolytes passing out in hypotonic solutions. If Fig. 194 be compared with Fig. 190, the difference between the polychaete and the echinoderm egg as regards the Boyle-Marriotte law will easily be seen.

A certain number of investigators have occupied themselves with the examination of the effects produced on developing eggs by environments of varying osmotic pressure. Morphological studies of this kind are fairly numerous, but the present summary is confined to those with a physiological bearing. Perhaps the most complete is that of Vlès & Dragoiu, who introduced the conception of "pression

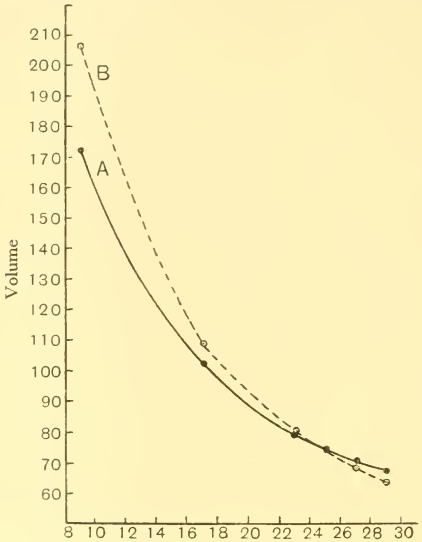


Fig. 194. A, experimental; B. theoretical.

osmotique d'arrêt". They thought that, if one could determine the osmotic concentration at which development ceased, it might be possible to calculate the "energy of segmentation" or of development itself. I shall return to this notion in the section on Energetics; here only that part of the work which relates to osmotic pressure will be considered. Placing the fertilised eggs of *Strongylocentrotus lividus* in solutions of glucose in sea water, they made a statistical study of the effects produced. From 25 to 30 atmospheres, the effect was almost inappreciable, perturbations of development

being almost absent, and a delay in cleavage not exceeding 15 minutes occurred. From 30 to 55 atmospheres there was a critical zone; the percentage of eggs achieving complete first division rapidly diminished until it reached a figure between 5 and 10. From 55 to 100 atmospheres, the state of affairs changed, for now, not only would cleavage not take place, but a great number of abnormal and teratological forms appeared. Later experiments gave greater regularity, and the same process was gone through for each of the early stages, i.e. second cleavage, third cleavage, fourth cleavage, etc. The result was that for all the stages averaged, it took an osmotic pressure of 33 atmospheres to stop 10 per cent. of the eggs developing, and 39 atmospheres to stop 90 per cent. of them. Therefore an osmotic pressure of about 11 atmospheres more than that of sea water was required to stop development. Vlès & Dragoiu went on to calculate the work done in elevating the osmotic pressure to this point, and hence "the external osmotic work of cytoplasmic division",

$$W = \omega_0 V_0 - \omega V,$$

where $W$ is the work, $\omega$ and $\omega_0$ the normal and stoppage osmotic pressures, and $V$ and $V_0$ the normal and stoppage egg-volumes. The values for $W$ fell with development in a straight line, as follows:

| | Ergs |
|---|---|
| First cleavage ... ... ... | 4·09 |
| Second cleavage ... ... | 2·05 |
| Third cleavage ... ... | 0·85 |
| Fourth cleavage ... ... | 0·29 |

Vlès & Dragoiu pointed out that the "travail d'arrêt" diminished as the volume of the blastomere diminished, but also tended to diminish when related to the volume of the egg as a whole. Chevroton & Vlès had previously accurately measured the volumes of the blastomeres, finding, for instance, that the two were not quite halves and the four not quite quarters. The following figures were obtained:

| | Volumes (Chevroton & Vlès) ($\times 10^{-8}$ c.c.) | "Travail d'arrêt" (Vlès & Dragoiu) (ergs) |
|---|---|---|
| Unfertilised egg ... ... ... | 52·3 | — |
| Fertilised egg (membrane) ... | 109·0 | — |
| ,, (egg only) ... ... | 47·7 | 4·02 |
| ½ blastomere ... ... ... | 17·3 | 1·66 |
| ¼ blastomere ... ... ... | 7·78 | 0·81 |
| ⅛ blastomere ... ... ... | 2·64 | 0·28 |

Vlès & Dragoiu also made a cytological examination of the material, and plotted the time taken to produce a definite morphological event such as formation of accessory aster, or "paquet chromatique",

against the osmotic pressure of the solution. The resulting graph showed the usual descending curves, for the higher the osmotic pressure the more quickly the abnormalities appeared, but in one case a complete curve was drawn through only two points, and in two cases through only one point—happily an unusual way of presenting facts.

Spaulding's work on the energy of segmentation, though it was earlier and based on excellent theoretical principles, was very similar to that of Vlès & Dragoiu. By immersing echinoderm eggs in solutions of differing osmotic pressure, he was able to find the solution which just stopped cleavage, thence the internal osmotic pressure, and thence the energy in ergs required to stop cell-division. The osmotic pressure sufficient to inhibit the first segmentation, and therefore equal to the resultant internal pressure, was 7·32 atmospheres, for the second segmentation 6·53, and for the third 6·40. From this he calculated that the energy required to stop the first division was 1·567 ergs, and that required to stop the second one was 1·399 ergs. 'This means", he said, "that, as involved in or as identical with the first segmentation, there has been a resulting energy decrease, therefore, of 0·168 erg, or that it has taken this amount of energy, about 1/9 of the total increase resulting from fertilisation, etc., to bring about this cleavage." Similarly, to bring about the second cleavage, 0·028 erg was involved.

The conclusions of Spaulding and of Vlès & Dragoiu were for a time generally accepted, but the conception of "travail d'arrêt" has now, largely owing to the criticisms of Rapkine, fallen into disrepute, for it rests on a fallacy, assuming as it does that it would be possible to find the work done by a man in walking a mile by measuring the work done in stunning him with a stick or a stone at the end of it. "In order to use the 'travail d'arrêt' as a measure of the 'travail de division' the phenomena would have to be reversible, i.e. capable of coming to equilibrium, but that is just what they are not." (Rapkine.)

Bialascewicz approached the problem in another way, estimating the relative speed of development of embryos in solutions of different osmotic pressures. Working with *Echinus microtuberculatus*, *Strongylocentrotus lividus* and *Rana fusca*, and using hypotonic and hypertonic sea water in the two first cases, and glucose in the third, he found that the maximum speed of development took place in a medium isotonic with the normal medium, and on both sides of which the rapidity of development fell off sharply. These bell-shaped curves are shown

in Fig. 195. In the case of the frog, the curve appears to drop only on one side. The zone within which normal segmentation would proceed also differed markedly for the two echinoderms; for instance, it was $\Delta - 0{\cdot}63°$ in the case of *Echinus* and only $-0{\cdot}57°$ in the case of *Strongylocentrotus*, while in the case of *Rana* it was only $-0{\cdot}17°$. On the other hand, the zone between the osmotic pressures which caused death was relatively wider in the case of the amphibian than in the case of the two echinoderms. The effect of osmotic pressure on the embryos, moreover, was not the same at all stages. As regards mortality, the sensitivity of frog embryos to hypertonic solutions augmented with age; thus at the 8-cell stage they were killed in 24 hours by solutions of $\Delta - 0{\cdot}95°$, but at the time of hatching they were killed in 24 hours by a solution of only $-0{\cdot}43°$. On the other hand, the embryos of *Strongylocentrotus lividus* supported changes of osmotic pressure better the older they were. As regards speed of development between given stages, this also was affected differently. As is seen from Fig. 195, between the 4-cell stage and the appearance of the first mesenchyme cells, the speed of development falls off distinctly less slowly from the optimum with change of osmotic pressure than it does between the appearance of the first mesenchyme cells and the three spicules of the pluteus.

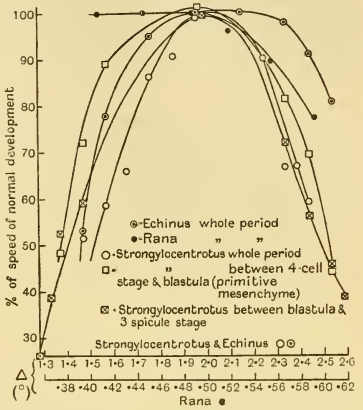


Fig. 195.

Fauré-Fremiet made parallel experiments on the eggs of *Sabellaria alveolata*, and obtained precisely similar results. Fig. 196 taken from his paper shows the usual bell-shaped curves, resulting from the plot of active osmotic concentration against the time required to reach certain stages of development. There is a certain optimum osmotic pressure, from which the speed of development rapidly falls away on both sides; thus, although the processes of division will go on between the limits of 0·85 and 1·48 mol. per litre, the

optimum is at 0·956 mol. per litre. Mortality also shows a bell-shaped curve, with an optimum at 1·024. Fauré-Fremiet concluded that as the Boyle-Mariotte law $pV = K$ holds for the egg of *Sabellaria alveolata*, the method of calculation of Vlès & Dragoiu is not applicable to it. He noted finally that, at external osmotic pressures which greatly slowed down the speed of development, the

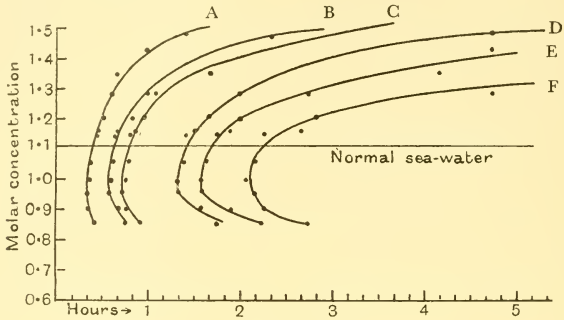


Fig. 196.

A, 1st polar body. B, 2nd polar body. C, Cordiform stage.
D, Stage II. E, Stage IV. F, Stage VI.

processes of division, etc., were not appreciably modified. In another work he stated that the outer egg-membranes of *Ascaris* were absolutely impermeable, the surface of the egg cytoplasm only permeable to water, and that the egg-contents was isotonic with a 0·7 per cent. sodium chloride solution.

## 5·6. The Osmotic Pressure of Terrestrial Eggs

The eggs of terrestrial animals have not received as much attention from this point of view as have those of aquatic animals. A little work has, however, been done on the silkworm embryo. Polimanti in 1915 and Pigorini, Tonon, Tona & de Ziller in 1927 obtained the following figures:

| | Polimanti Δ (°) | Pigorini *et al.* Δ (°) |
|---|---|---|
| Ovarian eggs ... ... ... ... | — | −0·630 |
| Newly laid eggs (yellow) ... ... ... | −0·650 | −0·580 |
| After 2 or 3 days (ash-yellow) ... ... | −0·660 | −0·700 |
| After 3 or 4 days (quite ash-coloured) ... | −0·680 | −0·700 |
| After some months, i.e. in the torpid state | −0·690 | — |
| Caterpillar newly hatched ... ... | −0·665 | — |
| Adult imago ... ... ... ... | −0·750 | — |

There would thus seem to be a gradually increasing concentration of osmotically active substances in the silkworm egg as it develops. Polimanti did not make any remark on it, but some possibility evidently exists that the fundamental mechanisms here are like those we have already seen to hold in the case of other arthropod embryos. It must be remembered, however, that the water-content of the silkworm egg is not constant, but decreases by evaporation from the time of laying onwards.

The investigations of the osmotic pressure of the constituents of the bird's egg before and during its development have been few in number, and not very complete. The first study was that of Atkins, who found that the osmotic pressure of the blood of the adult hen was two atmospheres greater than that of the fresh egg (mixed white and yolk).

| | Δ (°) | |
|---|---|---|
| | Adult blood | Egg |
| *Gallus* (hen) ... ... | −0·607 | −0·454 |
| *Anas* (duck) ... ... | −0·574 | −0·452 |
| *Anser* (goose) ... ... | −0·552 | −0·420 |

During incubation the osmotic pressure of the white and yolk mixed rose to about that of blood, a phenomenon at first sight like that seen in the amphibian and cladoceran egg, but probably partly due to the loss of water by evaporation, and partly to the inorganic salts entering the egg from the shell. Bialascewicz later went into the question in detail. His results are shown plotted in Figs. 197 and 198. It may be noted at a glance that the osmotic pressure of the embryonic body rises steadily as development goes on, that of the amniotic liquid stands more or less stationary, and that of the allantoic liquid greatly declines. What is the significance of these changes?

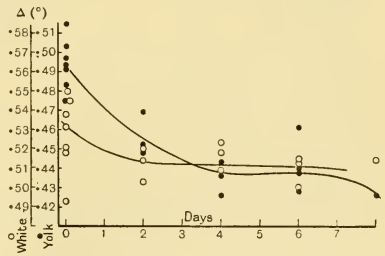


Fig. 197.

To begin with, the fact that the yolk has a distinctly higher osmotic pressure than the white may at any rate partially explain the passage of water from the white into the yolk, which has been noticed by so

many workers (Aggazzotti; Greenlee) during the first week of incubation. That it does not entirely account for it will be apparent when the work of Vladimirov and his collaborators is considered (see (p. 881). Then it must be remembered that all the points on the graph in Fig. 198 are at a lower level than the Δ of the blood of the adult hen, which Bialascewicz found to be – 0·635°. The yolk of ovarial eggs he found to be – 0·613°, and the yolk of eggs from half way down the oviduct – 0·585°, so that the falling curve for the yolk seen during the early days of incubation is simply the continuation of a curve which could be constructed for all stages after the yolk leaves the ovary. Thus, by the time the yolk has arrived at the stage of being laid, it is distinctly hypotonic to the parent blood, as is also the white, which has surrounded it. Processes of some sort, therefore, must be taking place, tending to diminish the concentration of osmotically active substances in the yolk. Bialascewicz supposed the yolk to absorb water as soon as it came into contact with the white in the oviduct, and he regarded the state of affairs at laying as an equilibrium condition, in which the osmotic pressure value was regulated by the degree of stretching which the elastic vitelline membrane could stand[1]. During incubation, the membrane became more extensible, and further dilution of the yolk by water from the white proceeded. Bialascewicz confirmed the old observation of Harvey that the white almost entirely disappears before the end of incubation, and gave a few figures,[2] but the most complete data showing this are some which I collected in

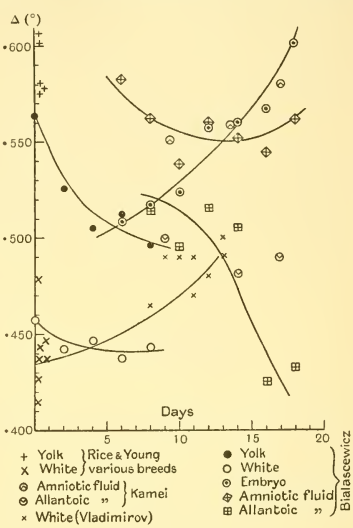


Fig. 198.

[1] The equilibrium may also, however, be chemical in origin (see p. 819). Bialascewicz had no evidence for his views on the elasticity of the vitelline membrane.

[2] See also Fangauf's data.

1927, and which are shown plotted in Fig. 199. The sharp descent of this curve between the 15th and 20th day must no doubt be due to the formation of what Duval called "the avian placenta". As the two ends of the allantois fuse at the sharp end of the egg they enclose in a vascular bag what remains of the egg-white together with a little yolk squeezed out through the incomplete closure of the yolk-sac. Absorption of the contents of the bag is thus greatly facilitated, so that by the time of hatching the only reserve of food left is the yolk itself. As the contents may be said to consist mainly of protein we should expect to find a marked peak on the curve relating absorption-intensity of protein to time, at this point, and as Fig. 250 shows, we do in fact find such a peak.

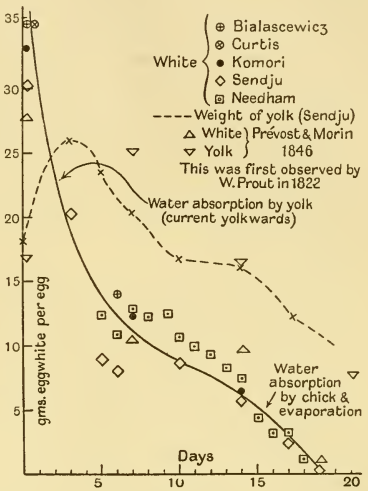

Fig. 199.

It is next important to note that the amniotic fluid is first of all hypertonic, then isotonic, and lastly hypotonic with respect to the embryo, which holds on its way steadily throughout development, until at the close of incubation it has almost reached the adult level. The embryo would seem to be independent of its surroundings, and to possess osmotically active substances in constantly increasing amount. It is interesting that chick embryo cells in tissue culture require an isotonic medium for long-continued growth (Ebeling) though they can support hypertonic and hypotonic media for some time. Hogue and Willmer found that osmotic pressure affected cell-migration but according to Lambert and Ebeling it has no effect on cell-multiplication. As for the allantoic fluid, the sudden fall of osmotic pressure towards the end of incubation was brought about, Bialascewicz suggested, by the functioning of the embryonic kidneys, excreting into it dilute urine of low osmotic pressure. Possibly the fall is an index of secretory activity of the metanephros. In all stages

the allantoic fluid is strongly hypotonic to the amniotic fluid, so that the embryo and amnion form a quite isolated system of high osmotic concentration, surrounded by a hypotonic allantois and yolk. In a certain sense, then, the amniotic fluid in the chick might be said to correspond with the perivitelline fluid in the frog embryo. The slight falling off in the osmotic pressure of the amniotic fluid which Bialascewicz found in the case of the chick, is paralleled by a similar fall in the case of the sheep (Jacqué), the cow (Grünbaum), the dog and the rabbit (Politi), and man (Ubbels). Again, Jacqué and Grünbaum found in mammals a rise, not a fall, of osmotic pressure of the allantoic fluid during development. Consideration of the mass of literature dealing with the osmotic pressure of the mammalian amniotic fluid, placenta, and foetal blood, will be deferred to the sections specially devoted to those subjects. However, the allantoic liquid in mammals also is always hypotonic to the foetal blood.

Bialascewicz pointed out that, in the period when the chick embryo was hypotonic to the amniotic fluid, it was not only not losing water, but rather was actually gaining it. His determinations of embryo water-content, however (see Fig. 220), were in a region which has been very little studied (i.e. before the 5th day) and it has been classical to believe that the embryo steadily loses water from the very beginning. As the discussion on p. 871 shows, however, Schmalhausen's data support those of Bialascewicz. The embryo becomes in turn isotonic and hypertonic with respect to the amniotic liquid, the water-content of which is indeed unknown, but must remain uniformly very high compared to the embryo.

Straub & Hoogerduyn were impressed by the very large difference existing between the osmotic pressures of the yolk (nearly $-0{\cdot}6^\circ$ $\Delta$) and the white (about $-0{\cdot}45^\circ$ $\Delta$) at the time of laying[1]. They devoted a long paper to the explanation of this fact, which is certainly remarkable considering how tenuous and fragile the yolk-membrane is. After considering all the possible systems which might be involved (Donnan equilibrium, etc.) they made some estimations of the constituents of the yolk and white (see Table 27) and drew up the following:

[1] At room temperature, this difference can be maintained for at least two months, i.e. quite as long as normal viability is retained (Moran).

| | Δ (°) white | Δ (°) yolk |
|---|---|---|
| K ... ... ... ... ... | 0·06 | 0·09 |
| Na ... ... ... ... ... | 0·04 | 0·15 |
| Cl ... ... ... ... ... | 0·08 | 0·13 |
| Lactate ... ... ... ... | 0·00 | 0·06 |
| Other monovalent anions ... ... | 0·02 | 0·06 |
| Free glucose ... ... ... | 0·05 | 0·00 |
| Unknown neutral substances ... | 0·17 | 0·08 |
| | 0·42 | 0·57 |

Although their figures for ash, especially in the yolk, do not agree altogether with those of other workers, yet their contention, that each dissolved substance varies quite independently in yolk and white, must be admitted. It is not merely that the total osmotic concentration of each phase is distinct but also that practically no agreement exists between the constituent items in that concentration. The difference between yolk and white is as much as 1·8 atmospheres, and if this was all due to the osmotic properties of the membrane alone, it would have to support, Straub & Hoogerduyn calculated, a pressure of 2 kilos per square centimetre. They considered that the Schreinemaker equations were therefore inapplicable, and as for those of Donnan, the distribution of ions in yolk and white was so different from what would be expected according to Donnan's theory that it was very unlikely it could hold in this case.

Straub & Hoogerduyn entered a quite new field when they suggested that the osmotic difference between yolk and white was a "Lebenswirkung" in the sense that the vitelline membrane might be physiologically bound up with the egg-cell or pre-gastrula. If this was so then after long storage the infertile egg should show a disappearance of the special membrane properties which characterise it when it is fresh. They mentioned, in this connection, the experiments of L. K. Wolff who had found that in the fresh egg there is a trace of zinc in the yolk and a trace of copper in the white while after storage for some time these metals are equally distributed throughout the egg. Their own experiments showed that eggs stored for a long time tend to acquire equal osmotic pressures on both sides of the membrane:

| | Δ (°) white | Δ (°) yolk |
|---|---|---|
| Fresh eggs ... ... ... ... | 0·45 | 0·60 |
| Conserved eggs ... ... ... | 0·50 | 0·52 |
| Frozen eggs ... ... ... ... | 0·49 | 0·50 |

They also found that if morphine, cocaine, or potassium cyanide was added to the egg-white in extremely small amounts (2·5 mgm. per egg) the difference in osmotic concentration between yolk and white could be much reduced, as if the membrane was no longer performing its function. Similarly, if yolk was put into a parchment capsule with egg-white outside, the system rapidly attained a state in which there was only a difference of 0·01° between the inside and outside freezing-points, instead of the 0·15° of the fresh egg. In another interesting experiment the yolk of a fresh egg was placed in diluted egg-white.

| | Δ (°) white | Δ (°) yolk |
|---|---|---|
| The yolk and white of the egg were at the beginning of the experiment ... ... ... ... ... ... | −0·46 | −0·58 |
| The yolk was then placed in egg-white which had been diluted with an equal quantity of water. Result ... | −0·21 | −0·58 |
| After 48 hours the system was ... ... ... ... | −0·23 | −0·38 |
| The yolk was then put back into natural egg-white. Result | −0·46 | −0·38 |
| After 48 hours the system was ... ... ... ... | −0·44 | −0·51 |

Thus in all the conditions the yolk maintained its hypertonicity, even when it had sunk to 0·38 and, at the beginning of the second period, was actually hypotonic to the natural egg-white. The "living" vitelline membrane must therefore tend to encourage the exit of water from the yolk or to impede its entry, on the one hand, and tend to encourage the entry of salts or to impede their exit, on the other hand.

If, then, the large difference in osmotic pressure between yolk and white was a "Lebenserscheinung" some energy-expenditure on the part of the egg-cell would be expected, and Straub & Hoogerduyn calculated the "Konzentrationsarbeit" required, to be 0·01 cal. per egg per day. Now there exist in the literature one or two papers which give the gas exchange and heat production of infertile eggs. A. J. M. Smith found that one unfertilised egg gave off 0·2 mgm. of carbon dioxide per day at 10° and Langworthy & Barott found that unfertilised eggs produced 0·01 cal. per kilo per hour at 12°, 0·02 at 15°, and 0·06 at 19°. Pucher studied the changes which take place in the incubated infertile egg during 20 days from laying. He found that the total glucose of the white fell by 90 mgm. per cent. and the total glucose of the yolk rose by 40 mgm. per cent. For purposes of rough calculation, the white may be taken as 33 gm. and the yolk as 16 gm., in which case the former loses 30 mgm. in 20 days, and the latter gains 6 mgm., so that the loss from the egg as a whole would

be about 24 mgm., or 1·2 mgm. per egg per day. Infertile eggs show, therefore, the following effects within 20 days after laying:

Loss of 0·2 mgm. carbon dioxide per egg per day (Smith).
Loss of 0·072 cal. per egg per day (Langworthy & Barott).
Loss of 1·2 mgm. glucose per egg per day (Pucher).

Smith's figure requires comment in view of the fact that eggs after leaving the hen, give off carbon dioxide to the environment, which is less saturated with that gas than the maternal body. Earlier work by Stepanek and by Atwood & Weakley (see Fig. 152), had resulted in values of the order of 10 mgms. of $CO_2$ per egg per day, but these were for the first week after laying. Smith's figure for the same period was about 3 mgms., but calculation shows that even this amount cannot be accounted for on the view that the egg is giving off the $CO_2$ which has been physically dissolved in it. It is necessary to postulate some acid in the shell liberating carbon dioxide from the carbonates there.

A steady level of 0·2 mgms. per egg per day, then, is reached by about a month after laying. The heat produced with this would be of the order of 0·37 cal., which would be more than ample to provide (*a*) for the heat output found by Langworthy & Barott, and (*b*) for the "Konzentrationsarbeit" of Straub & Hoogerduyn. The latter authors showed that their heat requirement could be satisfied by the daily combustion of 0·0025 mgm. of glucose. They also gave a theoretical excursus suggesting physical mechanisms by means of which this energy could be used at the membrane surface in the way they postulated. They regarded the vitelline membrane as a "galvanic combustion-element" for glucose with oxygen to carbon dioxide and water, so that the maintenance of specific concentration difference between the exterior and the interior would be a complicated case of concentration polarisation[1]. (For the histology of the membrane see Lecaillon.)

The egg of the pigeon (Riddle & Reinhart) and that of the mackerel (Alsterberg & Håkansson) show a very strong positive Manoilov reaction, which is probably to be interpreted as indicating a very weak metabolic intensity in the ovum before fertilisation.

[1] It was later shown by Hill, however, that infertile eggs kept in pure hydrogen for a month still retained the normal difference in osmotic pressure between their yolk and white. Whatever the mechanism of osmotic work may be, it can function anaerobically. In this connection the glycolytic power of the yolk, studied by Stepanek and by Tomita, should be remembered (see Sections 8·13 and 14·6).

The frequent occurrence of broken yolks in stored eggs shows that the water current eventually overcomes the resistance. Rice & Young estimated the osmotic pressure and the refractive index in the eggs of various kinds of hen, in order to assess the relative intensity of the water current in different eggs, but there were no perceptible variations from the mean. Kamei's data are in good agreement with those of Bialascewicz.

Table 92.

| | Osmotic pressure $\Delta$ (°) | | Refractive index at 20° C. | | |
|---|---|---|---|---|---|
| | White | Yolk | White | Yolk | Investigators |
| White Leghorn pullet | −0·435 | −0·580 | 1·3565 | 1·4175 | Rice & Young |
| White Leghorn hen | −0·442 | −0·601 | 1·3560 | 1·4188 | ,, |
| White Wyandotte | −0·428 | −0·576 | 1·3546 | 1·4183 | ,, |
| Barred Plymouth Rock | −0·436 | −0·602 | 1·3550 | 1·4192 | ,, |
| Rhode Island Red | −0·446 | −0·575 | 1·3568 | 1·4185 | ,, |

Various attempts have been made to gain some further information about the nature of the osmotically active substances in the yolk, as, for instance, Bialascewicz's own work on the electrolyte content of bird and fish egg-yolks, already mentioned in Section 1·16. In 1902 Stewart showed that hen's egg-yolk is a very much poorer conductor of electricity than a solution of its salts made up to the same volume. McClendon in 1910 examined the electrical conductivity of centrifuged suspensions of yolk, one poor in lipoid-protein yolk granules, the other rich in them. There was a slight difference between the two, the granule-poor suspension conducting rather better than the granule-rich one. Dilution, which breaks up ion-colloid compounds, made the conductivity of both suspensions decrease, a paradoxical result which McClendon was unable to explain.

## 5·7. Specific Gravity

The subject of osmotic pressure during embryonic life leads naturally to the discussion of specific gravity, many measurements of which have been made by marine biologists interested in the eggs of plankton. Here the question is complicated by the fact that the relative quantity of fats and oils, substances which have little or no osmotic activity, has importance in deciding whether an egg shall float or sink. The facts have been reviewed by Strodtmann and by Russell.

A good deal of our information about the specific gravity of marine eggs is inexact, for it is derived from the reports of those who have studied the frequency of the occurrence of forms of different developmental stages at different depths. Thus we know from the work of Holt that the eggs of the turbot *Rhombus maximus* sink rather quickly after the 7th day of development, and, according to Ehrenbaum, the eggs in the plankton sink as a general rule, for the lower the catch the more advanced the embryos. Raffaele, again, found that nearly all pelagic fish eggs get heavier as development proceeds, presumably because of loss of buoyant oily substances by combustion. Most eggs begin to sink at once, but one (that of *Labrax*) very slowly, and one (*Trachinus vipera*) only when development is half completed. Similarly Jespersen & Tåning observed that the larvae of the small oceanic fish, *Vinciguerria attenuata*, move down into deeper water as they develop (see also Yagle).

Indeed, as far back as 1897 Hensen & Apstein affirmed that the eggs of the cod, *Gadus morrhua*, sank regularly during development, for at the lower levels only the more advanced stages were found. This was denied by Hjort & Dahl, and by Kramp, but Jacobsen & Johansen (for *Gadus* and *Pleuronectes*) and Bowman confirmed it. The most recent investigations, those of Russell, show that eggs of *Gadus morrhua* and of *Sardina pilchardus* certainly sink, but those of *Clupea sprattus* seem to be equally distributed at all depths, and those of *Onos* are at all stages most abundant just under the surface of the water. Franz in 1911 supplied some quantitative data as shown in the following table, which demonstrated that at any rate for many eggs the specific gravity was higher at the end of the development than at the beginning.

Table 93.

| | Specific gravity | |
|---|---|---|
| | Beginning | End |
| Mackerel (*Scomber scomber*) ... ... ... | 1·0311 | 1·0317 |
| Gurnard (*Trigla gurnardus*) ... ... ... | 1·0307 | 1·0320 |
| Lemon dab (*Pleuronectes microcephalus*) ... ... | 1·0298 | 1·0306 |
| Turbot (*Rhombus maximus*) ... ... ... | 1·0307 | 1·0315 |
| Sprat (*Clupea sprattus*) ... ... ... ... | 1·0309 | 1·0312 |
| Tadpole fish (*Raniceps raninus*) ... ... ... | 1·0291 | 1·0309 |
| Cunner (*Ctenolabrus rupestris*) ... ... ... | 1·0296 | 1·0310 |
| Rockling (*Motella*) ... ... ... ... | 1·0297 | 1·0307 |
| Dragonet (*Callionymus lyra*) ... ... ... | 1·0320 | — |
| Sole (*Solea lutea*) ... ... ... ... ... | 1·0313 | 1·0341 |
| Dab (*Pleuronectes limanea*) ... ... ... | 1·0308 | 1·0339 |

In 1926 Spartà showed once more that most teleostean eggs have a rising specific gravity during development, but found that *Gobius jozo* was an exception, for the sp.g. of its eggs fell from 1·093 to 1·061. The course of the specific gravity has been charted out for a great variety of fishes by Remotti, some of whose curves are shown in Fig. 200. No satisfactory explanation of these phenomena has so far been given, but the experiments of Polimanti are rather suggestive. He estimated the fatty acid content of various fishes, getting the following results:

Table 94.

| | Fatty acids in % of dry weight |
|---|---|
| Pilchard (*Clupea pilchardus*) ... ... ... ... | 20·447 |
| Mullet (*Mugil chelo*) ... ... ... ... | 12·609 |
| Anchovy (*Engraulis encrasicholus*) ... ... ... | 9·133 |
| Scorpion fish (*Scorpaena scrofa*) ... ... ... | 7·211 |
| Mediterranean eel (*Congromuraena balearica*) ... | 6·768 |
| Blenny (*Blennius gattorugine*) ... ... ... | 6·422 |
| Torpedo (*Torpedo ocellata*) ... ... ... ... | 6·100 |
| Sole (*Solea ocellata*) ... ... ... ... ... | 5·448 |
| Dogfish (*Scyllium canicula*) ... ... ... ... | 5·315 |
| Weever (*Trachinus draco*) ... ... ... ... | 4·730 |
| Eel (*Conger vulgaris*) ... ... ... ... ... | 3·774 |
| Star-gazer (*Uranoscopus scaber*) ... ... ... | 2·600 |
| Sole (*Rhomboidyctis podas*) ... ... ... ... | 1·474 |
| Goby (*Gobius paganellus*) ... ... ... ... | 1·115 |

He then noted that this was also practically the order in which the fishes would be arranged, beginning with surface and descending to bottom fishes. He therefore suggested that the rise and fall of fish eggs during their incubation period was probably correlated directly with their changing content of fat. The influence of light on these ontogenetic vertical migrations must also be considered; indeed, according to Russell, it is the most important factor in them.

The eggs of fishes are not the only ones which move upwards and downwards in this way. Similar migrations have been observed in the cases of the chaetognath *Krohnia hamata* (Fowler), various copepods (Farran and Kraefft), the stomatopod *Squilla* (Santucci), and the siphonophore *Velella spirans* (Woltereck). Zahòny, who investigated the ontogenetic migrations of the chaetognath *Sagitta serratodentata*, regarded temperature and not specific gravity as the determining factor, but in support of Russell's view there are the laboratory experiments of Groom & Loeb (on the acorn barnacle, *Balanus perforatus*), and of Mast and Grave (on the tunicate *Amaroucium*), who all noted a changing reaction to light during the course of embryonic development.

Some curious experiments which may have a relation to these facts were made by Remotti, who caused the eggs of *Salmo lacustris* to develop, some in darkness, some in the illumination of a very powerful electric light. At the end of development the serum of the embryos which had developed in the light reacted more slowly and feebly with quinol than that from those which had developed in the

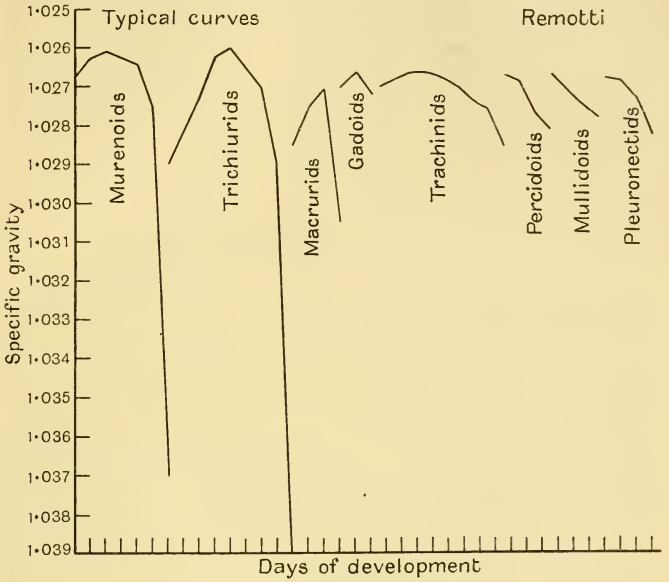


Fig. 200. Each curve begins at fertilisation.

dark. By the continuation of similar experiments Remotti hoped to identify in some way the factors responsible for the effect of light on developing embryos. For further discussion of this subject see Section 2·17.

Various other attempts have been made to unravel the mechanism responsible for ontogenetic vertical migrations. Sanzo reported, though without giving any figures, that the perivitelline liquid of murenoid eggs has a lower freezing-point at the end of development than at the beginning, i.e. that probably the membrane becomes

permeable to the salt of the environment as the embryo develops. This finding requires confirmation. Remotti has also studied the thermal expansion coefficient of teleostean eggs, which he claims differs slightly from that of sea water. He found that it definitely augmented during development.

The specific gravity of the amphibian embryo received a careful examination at the hands of Williams, whose data are plotted in Fig. 201. In all cases, there is a fall during development which is not interrupted at hatching. No doubt this is due to the absorption of water which is going on at that time (shown in Fig. 230). It must be remembered that the frog embryo is not separated from the yolk, so that the water absorption is not necessarily into the embryonic tissues. Williams also studied the centre of gravity in the frog embryo, finding that in the pre-hatching stages it was always at the cephalic end of the body, but afterwards, as the yolk was disappearing, it moved to the caudal end. Bialascewicz's figures for the specific gravity of frog larvae are in complete correspondence with those of Williams.

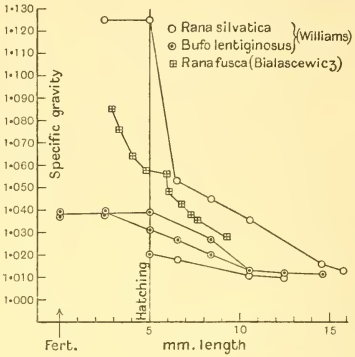


Fig. 201.

Hardly any investigations have been made of the specific gravity of the constituents of the hen's egg, except the very early one of Baudrimont & Martin de St Ange, who reported in their famous memoir of 1846 the specific gravity of various samples of yolk. The figures were as follows:

Table 95.

| | Specific gravity | | | Investigators |
|---|---|---|---|---|
| External albumen ... ... | 1·0399–1·0421 | Average | 1·0410 | Baudrimont & M. de |
| Internal albumen ... ... | 1·0421–1·0432 | ,, | 1·0426 | St Ange |
| Whole yolk well mixed ... | 1·0288–1·0299 | ,, | 1·0293 | Do. |
| Yolk taken from under the cicatricula, i.e. from the latebra | 1·0266–1·0277 | ,, | 1·0271 | Do. |
| Yolk taken from the opposite side ... ... ... ... | 1·0310–1·0321 | ,, | 1·0315 | Do. |
| Egg-white (all, well mixed) ... | — | ,, | 1·04028 | Rakusin & Flieher |

They felt, therefore, that they had explained why the yolk always floats with the germinal disc upwards, and they suggested that the oily substances were more concentrated there. This explanation is difficult to accept, in view of what we know about the relations between the white yolk and the yellow yolk. Baudrimont & Martin de St Ange drew a tentative parallel between the oriented floating of the hen's egg-yolk and the animal and vegetal poles of the frog's egg. Among the few pieces of work on this subject is that of Mussehl & Halbersleben and Dinslage & Windhausen who found that the specific gravity of different individual batches of eggs bore no relation to the percentage hatch. Groebbels has shown in the case of a number of wild birds' eggs that the specific gravity decreases during the course of development: in some instances this process goes exactly inversely to the weight of the embryo.

## 5·8. Potential Differences, Electrical Resistance, Blaze Currents, and Cataphoresis

Some further remarks must now be made on the subject of the electrical properties of embryos. A certain number of investigators have found a constant potential difference between the head and the tail ends of various embryos, and their work was reviewed by Hyman & Bellamy in 1922. Hyde's work on the potential of sea-urchin embryos may be mentioned—her experiments on frog embryos were confirmed by Viale.

More recently work on these lines has been carried on by Gayda. Leading off through electrodes placed at the poles of the embryos, he found the potential differences to be as follows:

| *Bufo vulgaris* | Hyde's figures (volts) | Gayda's figures (volts) | Gayda's figures (amperes) |
|---|---|---|---|
| Fertilised egg ... ... ... | — | $<3{\cdot}0 \cdot 10^{-5}$ | $3 \cdot 10^{-9}$ |
| Embryo ready to hatch ... | $2 \cdot 10^{-5}$ | $<5{\cdot}0 \cdot 10^{-5}$ | $3 \cdot 10^{-9}$ |
| Tadpole a week after hatching | | | |
| head–tail end ... ... | — | $21{\cdot}9 \cdot 10^{-3}$ | — |
| head–rump ... ... | — | $14{\cdot}5 \cdot 10^{-3}$ | — |
| rump–tail end ... ... | — | $5{\cdot}1 \cdot 10^{-3}$ | — |
| After metamorphosis | | | |
| head–rump ... ... | — | $13{\cdot}6 \cdot 10^{-3}$ | — |

More detailed figures are shown plotted in Fig. 202. It is very striking to notice the way in which the electrical resistance rose continuously. More examples of this phenomenon will shortly be given, and it seems to be, indeed, a general rule that the electrical resistance

of a tissue or of a whole organism increases with age. Whether this has any relation to the salt- or ash-content will be discussed later, but Gayda's figures for electrical resistance in Fig. 202 should certainly be compared with the figures for salt-content of frog embryos and tadpoles obtained in the work of Schaper, and plotted in Fig. 230.

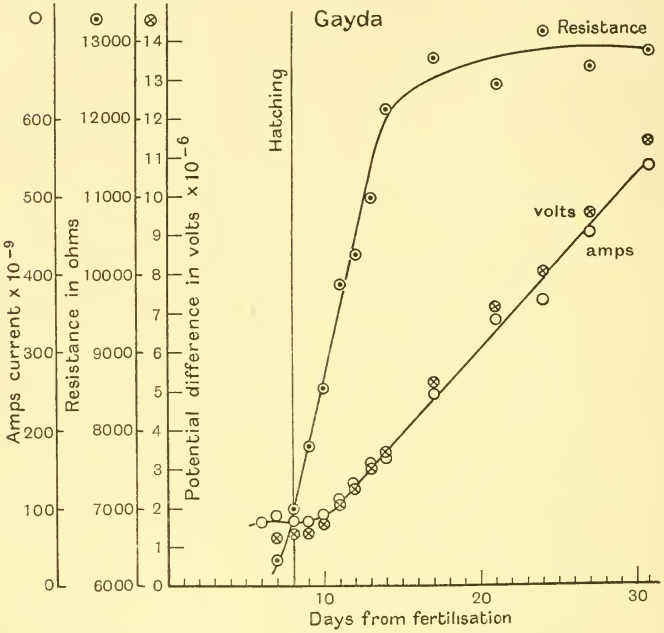


Fig. 202.

The time relations are astonishingly concordant, for, whereas according to Gayda the electrical resistance rises steadily until the 16th day from fertilisation, so according to Schaper the ash-content falls steadily until the 16th day after fertilisation. After that point the ash-content slowly rises again, and conversely the electrical resistance slowly falls, or remains constant. In Fig. 202, the potential difference between head and tail end of the embryos in volts and amperes should be observed, passing steadily upward on

the same curve. I have not figured Gayda's points for the period after the 30th day from fertilisation, for, with the initiation of metamorphosis, the embryological sphere is transgressed, but it may be said that the electrical resistance is gradually lowered, until at the time of completion of the posterior legs it has attained a practically constant value. This equates extremely well with the behaviour of the total ash as shown in Fig. 230. The amperage and voltage of the current between head and tail continue to increase more or less regularly through metamorphosis. The difference of potential between the tail end and the head end suffers an extremely rapid change over at about the 95th day from fertilisation, i.e. just before the completion of metamorphosis; before that time the tail end was always about $10.10^{-3}$ volts higher potential than the head end, but after it the reverse relation held. Gayda concluded that the embryo could be pictured as a series of solutions of diverse concentration and constitution, separated by semipermeable membranes[1], and that changes in morphology in such a system would have the effect of setting up small currents such as he had measured.

Mendeléef has concerned herself much with the comparative electrical resistance of tissues. Using Philippson's method for determining the electrical resistance of tissues, which consists in measuring the resistance a known amount of tissue opposes to the passage of an alternating current of frequency varying from 1000 to 3,000,000 periods per second, produced by a thermionic valve, she investigated the magnitude of Philippson's constants for embryonic and adult tissue. These constants are (*a*) $R$, the specific resistance of the cell-contents, i.e. the reciprocal of the electrolyte-content of the cytoplasm, (*b*) $r$, the resistance per cubic centimetre of tissue, i.e. inclusive of intercellular spaces, membranes, etc., and (*c*) $p$, the resistance in ohms per cubic centimetre of tissue corresponding to polarisation at a frequency of zero. For the liver of the normal guinea-pig, these were as follows:

| | Ohms |
|---|---|
| $R$ ... ... | 195 |
| $r$ ... ... | 1790 |
| $p$ ... ... | $4{\cdot}56 . 10^5$ |

During pregnancy, $R$ was lowered by not more than 5 per cent., $r$ by about 15 per cent. and $p$ by 50 per cent. Mendeléef concluded

[1] Responsibility for the resistance must probably be placed more on the membranes than on the ash-content.

that the resistance of the cell-interior was hardly affected, but that the resistance of the cell-membranes was definitely less than normal. When these experiments were extended so as to include embryos, the interesting figures shown in Table 96 were obtained. Throughout embryonic life, the resistance of the embryonic tissues is obviously less than that of the tissues of the maternal organism but, by the time that birth is reached, the two are at an equality, or the reverse relation may even be present. After the birth of the embryo the

Table 96. *Electrical resistance of liver cells.*

Mendeléef's figures.

| Length of embryo guinea-pig (cm.) | $R$ (ohms) | | $r$ (ohms) | | $p$ (ohms $\times 10^5$) | |
|---|---|---|---|---|---|---|
| | Foetal | Maternal | Foetal | Maternal | Foetal | Maternal |
| 2 | 123 | 185 | 870 | 1515 | 1·37 | 2·21 |
| 3 | 70 | 180 | 880 | 1310 | 1·37 | 2·04 |
| 4 | 115 | 180 | 1175 | 1710 | 1·69 | 2·45 |
| 5 | 160 | 180 | 815 | 1710 | 1·27 | 2·45 |
| 6 | 120 | 185 | 1200 | 2065 | 1·87 | 2·65 |
| 7 | 175 | 200 | 985 | 1670 | 1·68 | 2·00 |
| 8 | 155 | 175 | 1320 | 1585 | 2·04 | 2·32 |
| Term | 147 | 180 | 1380 | 1300 | 2·01 | 1·90 |

maternal liver tissue returns within 48 hours to its normal level. Mendeléef concluded from all this work that the membrane permeability as well as the electrolyte concentration in the cytoplasm were higher in the embryo than in the adult, and probably higher the younger the embryo. We have had already a good example of the increase of electrical resistance with age in the work of Gayda (see Fig. 202). Evidently the electrolyte-content of a tissue or an embryo is not measured by its ash-content, yet it is not without significance, perhaps, that the ash-content of the chick embryo (see Fig. 402) and of the frog embryo (see Fig. 230) decreases with age. A striking illustration of this is provided by Fig. 249, which shows the behaviour of the inorganic substance/organic substance ratio for the chick embryo, a ratio which falls steadily from the beginning of incubation till the end. Mendeléef laid special emphasis on the importance of the placenta in separating two organisms with very different cellular permeabilities. She went on to study the effect produced by keeping the extirpated tissues *in*

*vitro* for some time. If the measurement is made three-quarters of an hour after the removal of the cells from the body, a much higher resistance is found than if it is made immediately after removal. This augmentation *in vitro* she found to be due only to $r$, not to $R$ or $p$; in other words, to an increasing ionic membrane impermeability. The cells do not vary in electrolyte-content, or in membrane polarisation. The augmentation is stopped by cold, and is therefore probably chemical; some tissues show it more intensely than others. In the case of the embryo, the following results were obtained:

Table 97. *Electrical resistance of liver cells.*

| Length of embryo (cm.) | $r$ (ohms) at the time of removal of the material | | 45 minutes afterwards | | Percentage augmentation | |
|---|---|---|---|---|---|---|
| | Foetal | Maternal | Foetal | Maternal | Foetal | Maternal |
| 3 | 909 | 1457 | 1453 | 3702 | 60 | 154 |
| 5 | 1240 | 1860 | 1986 | 4217 | 60 | 122 |
| 6 | 1425 | 1071 | 1756 | 3590 | 24 | 111 |
| 8 | 1140 | 1840 | 1300 | 3232 | 14 | 76 |
| Term | 1252 | 1233 | 2602 | 2790 | 108 | 126 |

Evidently the electrical resistance of the embryonic tissues does not augment *in vitro* to the same extent as that of the maternal tissues, at least until birth, by which time the two react very similarly. Mendeléef concluded that there was a relation between decrease of membrane permeability *in vitro*, and its absolute level in growing and stationary tissues.

Philippson's methods (much modified) have also been used by Cole to determine the impedance of suspensions of eggs to various frequencies of alternating current. Operating on the eggs of *Arbacia punctulata* Cole found that before fertilisation the values of the impedance for any given frequency were quite variable, and there were similar variations in the average specific resistance of the egg at both high and low frequencies. Immediately after fertilisation, however, these quantities became quite constant and did not change noticeably thereafter. Cole did not regard the work of Gray and of McClendon, mentioned above, as very satisfactory, for it was done at low frequencies and in such conditions that almost all the current would flow through the intercellular sea water. In this way a very small change in the size of the eggs would cause a considerable change in the overall conductivity. Cole concluded, for

his part, that the specific resistance of the interior of the egg was about 90 ohms per c.c. or 3·6 times that of sea water, and that the impedance of the surface of the egg is probably similar to that of a "polarisation capacity". On these values membrane formation and cell division seemed to be without effect.

The electrical resistance of the constituents of the hen's egg has been studied by Bellini. The figures he obtained are shown in Fig. 203. If the egg is not incubated at all, the resistance of the yolk and the white remains quite constant even over a much longer period than that represented on the graph. If, however, the egg is placed at 37°, the resistance of the yolk augments until the 6th day, after which it declines to its initial value. That of the white was not followed by Bellini after the 8th day, but rose quickly up to that point. It is not easy to interpret these results, but presumably they indicate a demobilisation of free electrolytes throughout the extra-embryonic part of the egg during the 1st week of development. Practically nothing is known about the behaviour of the inorganic constituents of the yolk and the white during this time[1]. The fact that the embryonic body is at this stage more rich in ash, and therefore probably in electrolytes, than at any other stage, though unknown to Bellini, may have some bearing on the question, but the embryo is now so small in relation to the yolk and white that it is doubtful whether its absorption of electrolytes could account for the changes in electrical conductivity of the rest of the egg.

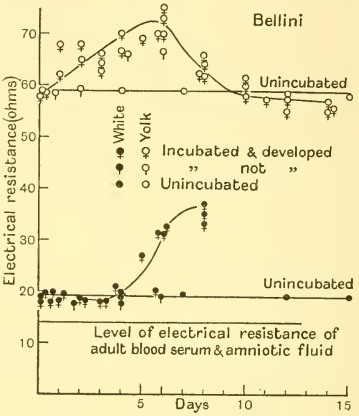


Fig. 203.

According to Fürth the dielectric constant of yolk in the hen's egg is 60 and that of white 68.

Other phenomena were discovered by Hermann & von Gendre, who found in 1885 that the developing chick embryo is always positive to the yolk and the white. At 80 hours' development, there

[1] But see Fig. 405 and Section 13·1.

was a maximum E.M.F., but they were not able to offer any explanation of this, nor have any more recent workers gone into the matter anew. Their figures, which are presumably to be explained on a concentration-cell basis, are plotted in Fig. 204. Waller investigated the "blaze currents" of the developing hen's egg. He had previously defined a blaze current as an electrical response to some kind of stimulus, whether electrical, chemical or photic. In its most characteristic form it occurred in the same direction as the current by which it had been excited, and this was important, for it could therefore not merely be a polarisation counter-current. The blaze current, according to Waller, is precisely analogous with the discharge of an electrical organ, excited by an electrical current in the homonymous direction. Having studied it in the eyeball and the crystalline lens, he turned his attention to the hen's egg, and, thinking that its presence or absence might be an indication of whether the embryo was living or not, he investigated a number of incubated eggs. As he had expected, the blaze reaction made its appearance as development progressed. Electrodes were brought into contact with the shell-membrane, a small piece of the shell having been removed, and whenever a blaze current appeared on stimulation, there a living embryo was found when the egg was opened, and *vice versâ*. Waller confirmed the observation of Hermann & von Gendre that there is a small current normally passing from egg-contents to embryo, and observed also that by repeated excitations the embryo can be killed or exhausted. Fig. 205 shows the decreasing blaze current obtained from an egg which was stimulated several times after 48 hours' incubation. Waller found that in the early stages, before the blastoderm membrane had folded to form a tubular embryo, the blaze currents were always positive, no matter whether the excitation was positive or negative, but later the blaze currents were always homodrome with the direction of excitation, positive if the excitation was positive, negative if it was negative. He extended these observations to frog's eggs, and reported that for the most part definite blaze currents had been given by them.

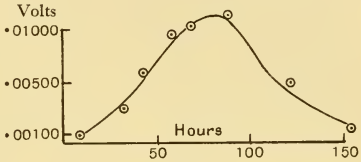

Fig. 204.

Not a few researches have been made in which the effect of electrical energy applied in various ways has been tested as a stimulus for embryonic growth or differentiation. The results have been uniformly negative, except from a teratological point of view, and even then very little illumination has resulted from such experiments. In 1840 Rusconi initiated this type of work by exposing hen's eggs to the action of an electrical current, constantly flowing, but his results were quite negative. Fasola in 1887 and Roux in 1889 both found nothing but a few malformations, which any treatment might have produced. In the last decade of the last century, there were several

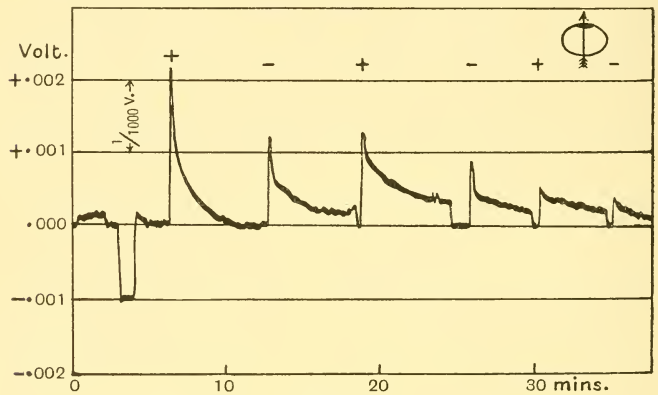


Fig. 205.

papers on this subject; thus Windle stated that a slight retardation of growth was noticeable when the embryo was subjected to a continually flowing electric current, but that incubation in a strong magnetic field was favourable. His experiments were not done statistically. Dareste obtained teratological effects, but was contradicted by Pierallini, and Rossi stated that development was undoubtedly modified by electric currents, but probably only indirectly on account of interference with other factors. The only recent investigations of the matter are those of Gianferrari & Pugno-Vanoni, who in 1923 reported the results of subjecting salmon eggs to high frequency currents during their development. Duplications and various terata were produced. On the whole, this seems to be

a very unpromising line of research, for, to begin with, it will always be a matter of extreme difficulty to hit on the right strength of current or of magnetic field to influence the embryonic processes without causing pathological states to arise. It is interesting, however, that Brown found that the eggs of *Fundulus* were as immune to electrical stimulation as to other influences, such as osmotic pressure.

Among the various electrical properties of eggs and embryos which are to be discussed in this section the charge on the egg has so far not been mentioned. Cataphoresis experiments with echinoderm eggs were made by McClendon in 1914, who found that, when the jelly had been removed from them, they were transported to the anode, and possessed therefore a negative charge. The surrounding jelly, however, went the other way, and had a positive charge; thus McClendon suggested that the fertilisation membrane might be the result of mutual precipitation by antagonistically charged colloids. Tomita, again, working with nematode eggs (*Ascaris*, *Oxyuris*, *Ankylostomum*), trematode eggs (*Distoma*) and cestode eggs (*Taenia* and *Bothriocephalus*), found in all cases a migration from cathode to anode, and so a negative charge. Szent-Györgyi, however, found no cataphoretic movement at all in the case of *Labrus rupestris* and *Echinus vulgaris* eggs. Vlès & Nouel, who made some experiments involving the degree of agglutination of echinoderm eggs at different *p*H, agreed with the conclusions of McClendon, although experimentally their *Strongylocentrotus* eggs moved towards the cathode. In view of the fact that three different workers have obtained the three possible results on this material, it is clear that further observations are to be desired. Vlès, Achard & Prikelmaier, working on the pounded and cytolysed protoplasm of *Strongylocentrotus* eggs, found cataphoresis towards the cathode below *p*H 5·8 and towards the anode above it, from which they concluded, as has already been mentioned, that the iso-electric point of the egg-proteins was about 5·5.

## 5·9. Refractive Index, Surface Tension and Viscosity

Vlès has studied a number of the biophysical properties of the sea-urchin's egg, such as refractive index. He regarded the refringent spherical or ellipsoidal egg as a lens, from which the refractive index could be calculated, knowing the focal distance. The necessary measurements were (1) the curvature of the egg, ascertained by micrometric measurements along different diameters, (2) the focal

distance, measured optically. In a second paper he studied the change in refractive index during the period from fertilisation to first cleavage in the sea-urchin's egg. A small fall after fertilisation led to a large rise after the appearance of the diaster, and a rapid fall immediately before cleavage, the outside limits of refractive index within which these changes took place being 1·381 to 1·405. Their significance was doubtful.

The effect of heat on the physical properties of egg-cells has been studied by Achard. The volume, calculated from the diameter, attained a definite maximum at 35°, being $44{\cdot}5 \cdot 10^{-8}$ c.c. at 20°, 54·3 at 35°, and 46·0 at 41°. The specific gravity measured by immersion of the eggs in solutions of equal osmotic concentration but different density varied little, but had a slight peak at 36°. The electrical charge had a maximum at 35°. The oblateness (calculated from measurements of maximum and minimum diameter) had a maximum at 35°, and the surface tension (calculated from the oblateness) had a minimum at the same temperature. Achard concluded that the physical properties of these egg-cells could be interfered with to a slight degree apparently without preventing normal fertilisation and cleavage. The curves she obtained are given in Fig. 206, and show how 35° is in nearly all cases a critical point. The important point is that it is also a critical point biologically, for below it the percentage of eggs forming normal fertilisation membranes is uniformly 100, and above it falls off, while just the same applies to the percentage of eggs successfully accomplishing their first cleavage.

Vlès himself did a good deal of work on the surface tension of echinoderm eggs, treating them from the point of view of the physics of semiliquid drops, and examining their departure from the form of a perfect sphere under different experimental conditions. The surface forces acting on an egg can be evaluated, according to Vlès, from the degree of oblateness or flattening which the egg suffers when it rests on a horizontal surface under the influence of gravity. Unfertilised sea-urchin's eggs, devoid of jelly, show minima of flattening between *p*H 3 & 5 and 8 & 10. The tensions involved are of the order of 10 to 25 dynes per centimetre.[1] After fertilisation the surface tension is reduced; it rises again before the fusion of male

[1] A much lower value (1·3 dynes per centimetre) is obtained for *Chaetopterus* eggs by observing their fragmentation in the microscope-centrifuge (Harvey & Loomis; Harvey).

and female nuclei, and falls when that process is complete. It rises again to the diaster stage, and diminishes markedly immediately before cleavage. The second augmentation would correspond with the semipermeable phase of Herlant and the period of augmentation of

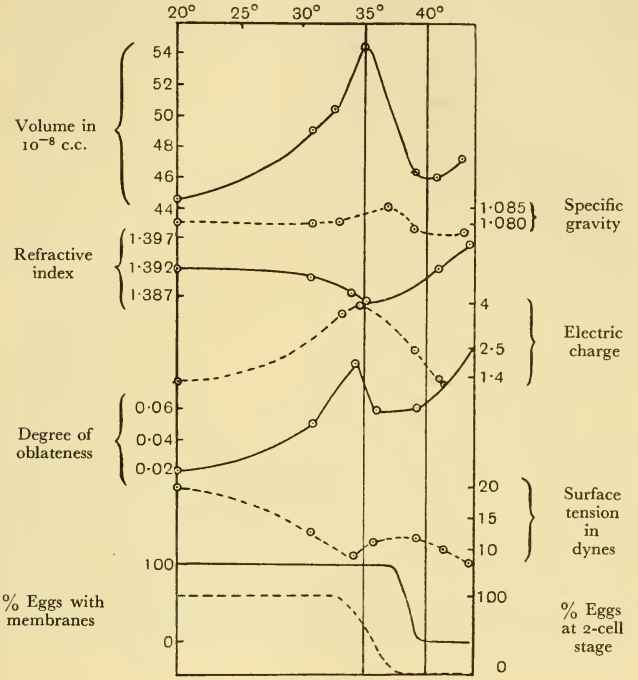


Fig. 206.

refractive index found by Vlès. The curves with which this paper is illustrated seem to indicate an unwarranted confidence in the accuracy of individual points, and the number of observations is hardly sufficient to establish some of the conclusions.

The subject of protoplasmic viscosity has attracted a very great number of investigators, and cannot be reviewed here, but certain work of special embryological importance must be mentioned. In

the first place, Bellini has studied the viscosity of the yolk and the white in the hen's egg during the early part of its development, using Fano & Rossi's viscosimeter. The values he obtained are shown in Fig. 207. Evidently if the eggs are not incubated the viscosity of the white increases steadily but slowly, and that of the yolk decreases at much the same rate. If the eggs are incubated, the viscosity of the white increases more rapidly, and that of the yolk decreases far more so, descending from 30 units to less than 1 by the 6th day of development. This reciprocal interchange of viscosity between the yolk and white evidently goes on irrespective of the embryo, though it is much accelerated by the presence of the latter, and Bellini rightly concluded that it was the result of a continuous dilution of the yolk from the water of the white. I shall return to this point in the succeeding section, when the whole question of water metabolism in embryos is being discussed, and though, as we have already seen, the osmotic pressure of the yolk is much superior to that of the white, Vladimirov's work has made it very unlikely that this can be the sole cause of the flow of water. Again, the viscosity of the white increases by only 4 units in the first 10 days, and that of the yolk decreases by as much as 30 units, so Bellini envisaged other processes than the dilution of the yolk by the water from the white as playing some considerable part in the decline of yolk-viscosity. It is known that the yolk contains an abundance of enzymes, which the incubation at 37° might allow to act, e.g. proteases, lipases; diastases (see Plate XI). Experiments that Bellini did with incubated infertile eggs showed that the great decline of yolk-viscosity did not go so far as with developing ones, but on the other

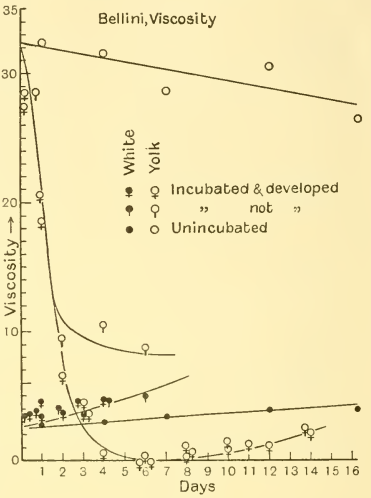


Fig. 207.

YOLK OF HEN'S EGG AT THE ELEVENTH DAY OF INCUBATION

Stained with iodine, showing its very heterogeneous character half-way through development. Note especially the large vacuoles filled with fluid contents. Magnification 6 × A, prepared and microphotographed by Dr V. Marza.

hand there was little difference between the viscosity of the white in incubated fertile and infertile eggs. The whole question merits more study, in view of the obvious importance possessed by the mechanism of preparation of the first pabulum of the embryo. For the frog's egg, rhythms of viscosity have been reported by Odquist, who, however, used a centrifugation method followed by observation on pigment distribution.

As regards the protoplasmic viscosity of alecithic eggs, Heilbrunn has worked on those of the clam, *Cumingia*, and the sea-urchin, *Arbacia punctulata*, using the centrifuge method. In both cases there was a maximum viscosity at 15°, from which the values fell away on both sides. Pigorini subsequently obtained very similar results for the expressed juice of silkworm's eggs.

Heilbrunn in 1920 also studied the effect of anaesthetics upon viscosity in eggs, and in 1915 the effect of hypertonic solutions; the original papers must be consulted for the details.

Seifriz has also done much along these lines, using for the most part the method of microdissection. In many cases this method has given diametrically opposite results to those obtained by other techniques, especially the centrifuge, and has occasioned some polemics which need not be described in detail. Seifriz maintained that the protoplasm of *Echinarachnius* eggs was immiscible with water, and allotted a definite degree of viscosity to the cytoplasm at different stages during cleavage, etc. A large element of the subjective must have entered into the determination of these values, and where they have conflicted with those obtained by the centrifuge method they have been abandoned by most biologists, who prefer the more objective technique. Seifriz's viscosity values were as follows:

| *vv* | % gelatine | Substances of equivalent viscosity |
|---|---|---|
| 1 | 0·0 | Water |
| 2 | 0·05 | — |
| 3 | 0·2 | — |
| 4 | 0·4 | — |
| 5 | 0·5 | Paraffin oil |
| 6 | 0·6 | — |
| 7 | 0·7 | Glycerol |
| 8 | 0·8 | Bread-dough |
| 9 | 1·0 | Vaseline |
| 10 | 2·0 | Firm gelatine gel |

On this scale the *vv* of mature unfertilised eggs of *Echinarachnius* and *Tripneustes* was 7. This agreed with the microdissection estimates

of Kite and of Chambers, who had stated that the ooplasm was just viscous enough to put a stop to Brownian movement. During mitosis the marked changes in viscosity led to a local lowering of viscosity to *vv* 3 or thereabouts. Seifriz's later experiments were very ingenious. He introduced by the aid of a micromanipulator a minute nickel ball about 7$\mu$ in diameter into the cytoplasm of the egg of the sand-dollar, *Echinarachnius parma* (a principle that had been adopted in the work of Freundlich & Seifriz on inorganic colloids). The particle having been introduced, it was attracted by a powerful electromagnet, until the colloid was stretched a certain amount. If this amount was not exceeded the particle would return to its original position when the current was cut off. The distance over which the particle travelled furnished a measure of the stretching capacity of the colloidal substance, and the force necessary to produce the stretching was a measure of the elasticity. It was found that a particle introduced into the protoplasm of an *Echinarachnius* egg could be moved backwards and forwards through the inner parts by the electromagnet, apparently without any injury being done, but that, when it came up against the cortical parts of the egg, its motion was much slower and might completely cease. Obviously their viscosity was greater. Quantitative calculations showed that the *vv* of the inner part was just the same as that which Seifriz had previously allotted to other marine eggs, i.e. barely that of concentrated glycerine (sp.g. 1·2500). The stretching distance of the cortex protoplasm was 9 $\mu$. Nothing has since been done to follow up this interesting type of investigation, but various investigations of viscosity in echinoderm eggs have been made (Barth ; Jacobs ; etc.) and for further information the monograph of Heilbrunn should be consulted.

## SECTION 6

## GENERAL METABOLISM OF THE EMBRYO

### 6·1. The *p*H of Aquatic Eggs

It will be convenient to take first the work on the single egg-cell, which has mostly been done on the eggs of marine invertebrates, and then to go on to the experiments which have dealt with the tissues and surrounding substances of such an embryo as that of the chick. Seven principal methods have been used in studying intracellular hydrogen ion concentration: (1) vital staining, (2) micro-injection of dissolved indicators, (3) micro-injection of solid indicators, (4) "micro-écrasement" or microcompression, (5) electrometric measurement by means of micro-electrodes actually in the cell, (6) electrometric measurement upon thawing crushed masses of eggs, (7) contact of indicators with crushed tissues. Each of these methods suffers from certain disadvantages. A critical comparison of them has been made by Reiss in his monograph on the subject, but his views, which are substantially those of the Strasburg school, have not been generally accepted. Vital staining is probably the least valuable of all the methods, for the dye may not penetrate and show a colour in the cell-interior until the cell has become completely abnormal[1]. Yet this, of course, was the general technique employed by the earlier workers, who would have preferred no doubt to use a pigment already naturally in the cells, as had been done in other cases, if eggs containing a natural indicator could have been found. The first observations were made by Schücking in 1903, who on inadequate basis regarded the granules in echinoderm eggs as alkaline and the protoplasm as acid. Then Loeb in 1906 stained sea-urchin's eggs with neutral red, expecting on theoretical grounds to observe a trend towards the acid side after fertilisation. This he failed to do, the dye absorbed by the eggs of *Strongylocentrotus purpuratus* from 1/100 molecular solution being quite red both before and after fertilisation. He noticed that after remaining 20 minutes in ordinary sea water, the unfertilised eggs became colourless, but the fertilised ones retained their colour. Parthenogenetic eggs gave the same effect. He also noted that the more development

[1] And even if it does, it may undergo chemical change at the cell-surface.

proceeded the deeper the eggs stained, and the more difficult it was to wash the stain out. He concluded that the internal *p*H was on the acid side of neutrality, but not much below 6·0. Moreover Warburg; Harvey; and Herlant all found by staining with neutral red that the contents of the echinoderm egg was more acid than sea water. Still earlier work by Keeble & Gamble, who had succeeded in staining *Hippolyte* and *Mysis* eggs with litmus, had indicated an internal *p*H of between 4 and 3, but it is probable that the eggs so stained were dead.

Other work involving the staining method was even less illuminating. Fauré-Fremiet, exposing the eggs of the polychaete worm, *Sabellaria alveolata*, to the action of such dyes as Nile blue hydrochloride, Nile blue sulphate and brilliant cresyl blue, concluded that a *p*H of nearly 13 was reached at fertilisation. This extraordinary result was clearly due to the use of dyes which even under the best conditions are not good *p*H indicators, and which could not be trusted in a medium containing fatty substances, for which they have a special affinity. Lewis, again, growing embryonic fibroblasts on nutrient media to which indicators had been added, could not get any of them to stain until death occurred, whereupon they indicated a cytolysis *p*H of below 5·0.

The study of intracellular *p*H by vital staining received a considerable impetus in 1925 from the work of Rous and his collaborators, who employed the method largely on the whole organism in mammals. Using his method, Harde & Henri found the following values for the mouse:

| | *p*H |
|---|---|
| Uterine wall ... ... | 6·0–6·2 |
| Placenta ... ... ... | 7·2–7·4 |
| Embryonic skin ... ... | 5·6–5·8 |
| Maternal skin ... ... | 7·4–8·6 |
| Embryonic blood ... ... | 5·8 |

This last value was in good agreement with another obtained in a similar way by Mendeléef on guinea-pig's blood, which she found to be at *p*H 5·8, although the maternal blood was *p*H 7·4. The tissue fluid of the embryo (how prepared?) she found to be at *p*H 6·0.

It is, of course, doubtful as to what exactly is meant by intracellular *p*H or the internal *p*H of the egg. There must without doubt be numerous phases, perhaps of widely differing *p*H, and all that we measure directly by the various methods is the "*p*H globale" or overall *p*H of the cell. Lucke drew attention to the fact that, even

when a cell seems uniformly stained, the dye may yet be in close association with minute granules or globules, and may be registering their internal *p*H instead of that of the continuous phase in which they are. Lucke centrifuged the eggs of *Arbacia* and *Cumingia*, so that four layers appeared in them, a lipoidal one at the top, next a clear empty homogeneous protoplasmic layer, then a layer of small granules and at the bottom a layer of pigment granules. Such zoned eggs placed in 1/40,000 neutral red or brilliant cresyl blue solutions, took up the dye, but only as regards the granules. The clear layer and the lipoidal layer remained quite unaffected, unless they were exposed to the dye so long that they became granular, but that was an irreversible change leading to death. The centrifuged eggs were normal in that they would fertilise and develop into gastrulae, and Lucke concluded from his experiments that the tint given by a *p*H indicator in a cell was by no means a measure of the *p*H of its clear protoplasm, but rather of its granules. This must, of course, be admitted; nevertheless, the average overall *p*H of a cell is a constant of much interest, and worth investigating.

The method of crushing the cell or cells, and so admitting the indicator to contact with the cell-contents, is an old one, and a great deal of work has been done with it. Its obvious disadvantage is that it does not guard against the effects of cytolysis, but, on the contrary, actually involves them as part of its measurement[1]. Much work has been done by this type of method on eggs, especially by the Strasburg school. The earliest observation was that of Dernby, who squashed the eggs of *Strongylocentrotus lividus* in indicator solutions, and obtained a value of *p*H 6·5 for the interior. He did not, however, follow up this line of work, and the technique was elaborated in much detail by Vlès. Vlès' apparatus differed little from what the older microscopists used to call a "compressorium"; the egg of an echinoderm, for instance, was placed in it, surrounded by an indicator solution, the egg was then compressed or crushed by turning the screw, until the dye penetrated into the cytoplasm, upon which the pressure was suddenly released, and the outside of the egg washed with sea water, or colourless solution. Vlès found that the eggs of *Strongylocentrotus lividus*, studied in this way, were yellow to brom thymol blue and to bromcresol purple, but also yellow to methyl

[1] The same remarks apply to Tchakhotine's attempt to determine intracellular *p*H by injuring echinoderm eggs in indicator solutions with ultra-violet light.

red, which showed that the internal *p*H was between 5 and 6, with an average of about 5·5. No change appeared after fertilisation, and eggs in segmentation stages gave the same results as the unfertilised ones. Cytolysis, according to Vlès, led to a rise in *p*H, the dye at the edge of the cell becoming purple or blue, instead of yellow, a phenomenon which he attributed to loss of carbon dioxide. At the same time, Reiss published a paper on the internal *p*H of the nucleus as distinguished from the cytoplasm. Included in it were various determinations by the microcompression method on other eggs, which gave results as follows:

| Species | *p*H | Investigator |
|---|---|---|
| Sea-urchin (*Echinus*) ... ... ... ... ... | 4·3 | Ashbel |
| Sea-urchin (*Strongylocentrotus lividus*) ... ... ... | 5·5 | Vlès |
| Heart-urchin (*Echinocardium cordatum*) ... ... ... | 5·0 | Reiss |
| Red-currant ascidian (*Styelopsis*) ... ... ... | 4·7 | ,, |
| Sea-hare (*Aplysia limacina*) ... ... ... ... | 4·7 | ,, |
| Blood-worm (*Arenicola claparedii*) ... ... ... ... | 6·0 | ,, |
| Polychaete worm (*Spirographis*) ... ... ... ... | 6·0 | ,, |
| ,, (*Nereis*) ... ... ... ... | 5·8 | ,, |
| ,, (*Sabellaria*) ... ... ... ... | 5·0 | ,, |
| Gastropod mollusc (*Trochocochlea lineata*) ... ... | 5·2 | ,, |
| Clam (*Mya*) ... ... ... ... ... ... | 6·2 | ,, |
| Parasitic copepod (*Chondracanthus lophii*) ... ... | 4·8 | ,, |
| Spider-crab (*Maia squinada*) ... ... ... ... | 4·8 | ,, |

For the comparative measures on nucleus and cytoplasm he used the almost transparent eggs of *Echinocardium cordatum* before they lose their germinative vesicles. He reported that he found the nucleus to take on a deep mauve colour with bromcresol purple, while yet the cytoplasm was distinctly yellow. Bromthymol blue coloured the cytoplasm yellow, the germinative vesicle yellowish green, and the nucleolus bluish green. The same effects were seen with the unripe eggs of *Strongylocentrotus* and *Sabellaria*. Reiss stated that, although the cytoplasm of the egg-cell remained fairly constant with respect to the *p*H of the external medium, the nucleus was markedly affected by it, and its *p*H varied in harmony with it. The experiments which supported this view were done on the eggs of *Psammechinus milearis* and *Echinocardium*, but they have never been confirmed. Complicated results were also found to follow when anaesthetics were used, but these also have not been repeated, and need not be discussed.

Later, Reiss made a special study of the changes in internal *p*H taking place in the echinoderm egg during cleavage. He used an apparatus by means of which half the field of the microscope was made to correspond to variable *p*H indicator tints by means of a

series of hollow wedges containing indicator solutions interposed between the microscope and the light source. The accuracy of the method was thus claimed to reach 0·02 *p*H, but this estimate has seemed much too favourable to other workers on the same subject. With the aid of this apparatus, Reiss found rhythmical variations from *p*H 5·4 to 5·6 in the cell-interior during the first 250 minutes after fertilisation, i.e. during the first two or three cleavages, and he pointed out that these variations closely agreed with the rhythmical elimination of carbon dioxide found by Vlès (see p. 642), but as we have already seen, there is reason for doubting these latter results. Just as Vlès' rhythmical results were not confirmed by Gray, so, as will later appear, my wife and I were not able to find evidence in favour of the existence of those described by Reiss.

An alternative colorimetric method to that of microcompression is, of course, that of micro-injection, whether of dissolved or solid indicators. Vlès himself, in his first paper on intracellular *p*H, said, "The microdissection methods of Chambers in which one injects an indicator directly into the cells, would be evidently the best, but their difficulty hinders their general application". Actually the first intimations of the value of this method were contained in the work of Chambers, who in 1923 injected neutral red solutions into echinoderm eggs, and observed that the tint taken up was always pink, and never more than faintly orange. This direction of investigation was followed up a little later by my wife and myself in a series of papers (Needham & Needham). We injected the appropriate indicators into the eggs of *Strongylocentrotus lividus*, and found that, although bromthymol blue was always yellow in the cell, bromcresol purple was always purple. This was in contradiction with the results of the Strasburg workers with the microcompression method, as, of course, was our final result of *p*H 6·5 for the intracellular hydrogen ion concentration. On other eggs treated in the same way we obtained the following values:

| Species | *p*H |
|---|---|
| Sea-urchin (*Strongylocentrotus lividus*) (unfertilised) ... ... | 6·6 |
| ,, ,, ,, (fertilised) ... ... | 6·6 |
| Heart-urchin (*Echinocardium cordatum*) (unfertilised) ... | 6·6 |
| Starfish (*Asterias glacialis*) (unfertilised) ... ... ... | 6·6 |
| ,, ,, ,, (fertilised) ... ... ... | 6·6 |
| ,, (*Ophiura lacertosa*) (unfertilised) ... ... ... | 6·8 |
| Ascidian (*Ascidia mentula*) (unfertilised) ... ... ... | 6·6 |
| Polychaete worm (*Sabellaria alveolata*) (unfertilised) ... | 6·6 |

Thus the differences between the kinds of echinoderms were almost imperceptible, and the eggs of a polychaete worm and a tunicate agreed very well with them. All the figures were at least one *p*H unit higher than those obtained by the method of microcompression. We attributed this simply to the slighter degree of injury done to the cell by the micro-injection method. At about the same time Schmidttman, using a technique in which she introduced solid particles of indicator into the cells, reported a value of *p*H 7·6 to 7·8 for the mammalian egg-cell, taken from ovaries of rats, mice, rabbits, and cats.

This led to an extended controversy, the details of which cannot be given here, but may be found in the original memoirs. Vlès and his associates maintained that our readings had been insufficiently corrected, we maintained that the *p*H of the egg-cells studied was in the neighbourhood of 6·5, and that on cytolysis values identical with those obtained by him were found. We regarded cytolysis as an acid-producing process, and considered that his technique was not capable of demonstrating the *p*H of an uncytolysed cell. Thus on injecting bromcresol purple into an unfertilised *Strongylocentrotus* egg-cell, for instance, one sees what we called a "purple puff" which lasts for some seconds, then giving way to a greenish tint, which soon turns yellow. But on microcompression of such an egg, according to Vlès, the first colour seen in the cell is yellow or yellowish green, so that, in our opinion, Vlès was never observing uncytolysed eggs at all.

In support of our view that the intracellular reaction is near the neutral point, and that cytolysis liberates acid substances which interfered with Vlès' method, there are many observations. Since, as we have seen, Warburg has shown that the lactic-acid-producing mechanism is not peculiar to muscle, but exists in all tissues, the probability of lactic acid being produced in cytolysing eggs is very considerable. Again, any vacuoles would naturally be expected to burst when the cell is squashed, and, since Greenwood showed as long ago as 1894 that the food-vacuoles of protozoa are distinctly acid, there is obvious danger from that source. Recently Howland has found a *p*H of 4·3 in the digestive vacuoles of *Actinosphaerium*. But, further, the researches of Parat and his school have shown that the so-called Golgi apparatus is in all probability a series of intraprotoplasmic spaces, a vacuome, which contains an acid liquid, and is filled up by the metallic reagents usually employed

to demonstrate it. For *Triton marmoratus* egg-cells Parat gives a *p*H of 7·2–7·3 for the protoplasm, but of 6·8–6·9 for the vacuome: for *Ascidia mentula* egg-cells 6·8 and 5·0 respectively (vital staining). (Only four complete studies of the behaviour of the Golgi apparatus during embryonic development exist: Gatenby and Hirschler on *Limnaea stagnalis*, Nihoul on the rabbit, Parat on the nudibranch molluscs *Aplysia* and *Polycera*.) Finally, Drzwina & Bohn have described a phenomenon which may be called "infectious cytolysis". If a number of small animals, such as planaria, or of eggs, are placed in a small space, it is found that the cytolysis of a few sets all the rest cytolysing, and these workers found that a culture of *Convoluta* by its cytolysis lowered the *p*H of its culture medium from 7·0 to 4·4 in two minutes. Similar observations were made by Rebello and by Drastich. In our own experiments we observed that when amoebae cytolysed, their internal *p*H went down from 7·6 to about 5·0, but when the marine egg-cells cytolysed their *p*H descended further and more rapidly from 6·6 to about 4·5. This has since been confirmed by Reznikov & Pollack. On these grounds we concluded that our more alkaline figures were more accurate than Vlès' acid ones. We laid some emphasis on the fact that we observed no change in the intracellular *p*H on fertilisation, and that, although we micro-injected blastomeres up to the morula stage, we never found any perceptible variation during the cleavage period. The intercellular fluid in the 16-cell stage and the liquid filling the blastocoele cavity we found to be at least as alkaline as *p*H 7·3. This was almost exactly the same figure as one previously obtained for the blastocoele of *Pomatoceros* by Hörstadius.

Certain other points also emerged from our work on these marine invertebrate eggs. If the egg-cells of *Asterias* were fertilised with concentrated sperm suspensions, and afterwards kept in conditions of bad oxygenation, a high percentage of abnormal forms made their appearance, protruding bulbs of protoplasm, dumb-bell shapes, abnormal divisions, etc. But in all these cases the intracellular *p*H was normal, and remained so until cytolysis set in, when it was, of course, lowered to *p*H 4·5, or below. Again, in injections of the 2-cell stage in *Strongylocentrotus*, one blastomere would be quite coloured with the dye used, and would even cytolyse before its fellow showed any abnormality. There was no connection between them. Then we found that in acid sea water of *p*H 6·0 the eggs would remain for at

least 2 hours without showing any change in internal *p*H, maintaining independence thus against a change of 2·4 *p*H units in their environment.

Subsequent micro-injection work has uniformly confirmed our findings as regards intracellular *p*H. Rapkine & Wurmser injected dissolved indicators into the nucleus and the cytoplasm separately of the egg-cells of *Strongylocentrotus lividus* and *Asterias rubens*, and could not distinguish any difference between the *p*H. Both nucleus and cytoplasm were in the close proximity of *p*H 7·0. Chambers & Pollack, however, did obtain a certain difference between the nucleus and cytoplasm in the case of *Arbacia* eggs, getting values of 7·6–7·8 for the nucleus, 6·6–6·8 for the cytoplasm, and 5·4–5·6 for the *p*H of cytolysis. They found, just as we had, that cytolysed material in time assumes the *p*H of the surrounding sea water; an observation which probably explains the tendency towards alkalinity which Vlès had associated with cytolysis. Injury to the nucleus did not affect its *p*H, but the spherical nuclear remnant persisting after injury gradually assumed the *p*H of the environment. Curiously, an indicator for which the egg was normally impermeable could penetrate into it through a tear in the surface if the environment was more acid than normal. Perhaps the plasmalemma coat of the protoplasm does not form so completely in an acid medium.

Our observations on the *p*H of the blastocoele cavity were greatly extended by Rapkine & Prenant, who in 1925 followed the course of events in detail. To begin with, in the blastula of *Strongylocentrotus lividus*, the *p*H (ascertained by micro-injection of dissolved indicators) was between 7·0 and 7·3, but a little after gastrulation, as soon as the primitive mesenchyme cells appeared, it rose through 8·0, at which point the spicules were first formed, to 8·5, after which it gradually descended again to its original value of rather less than the surrounding sea water. It was evident that the rise and fall of the curve (shown in Fig. 208) was associated with the process of deposition of calcium for the spicules. *p*H 8 had already been noted by Prenant to be the most favourable hydrogen ion concentration *in vitro* for the deposition of calcite, and it is known that the spicules of echinoderms are of this mineralogical form. This work was repeated on the eggs of *Echinocardium cordatum*, with the result that a precisely similar curve was found. Rapkine & Prenant pictured a

selective absorption of the carbon dioxide produced by the organism as a whole by the mesenchyme cells for the manufacture of calcium carbonate, a process which might well be expected to make the liquid of the blastocoele cavity more alkaline than usual. Next Bouxin found that a fall of *p*H irrespective of the acid employed would produce in *Strongylocentrotus lividus* larvae a retardation of skeleton formation or a complete stoppage, or even if the *p*H was lowered below 6·4 a regression of spicules already formed. Rapkine & Bouxin accordingly micro-injected indicators into the blastocoele cavity at different external hydrogen ion concentrations, and in fact found that when the exterior *p*H went down to 6 that of the blastocoele cavity also went down, and almost as far, keeping above it, however, to the extent of two or three tenths of a *p*H unit. In the extreme cases there seemed, then, to be an actual solution of the spicule. Rapkine & Bouxin found that during this process the *p*H of the liquid in the blastocoele cavity was maintained rather stable at *p*H 6·25, and they suggested that the regression of the skeleton might to a certain extent be considered as a defence mechanism in view of the fact that death invariably occurred when the blastocoele *p*H had reached 6·0. In the case of younger embryos, where the mesenchyme cells had just appeared, a small fall in external *p*H causes a retardation or an inhibition of skeleton formation, while the *p*H remains at about 7·4; thus the mesenchyme cells are removing carbon dioxide, but the external acidity prevents the *p*H rising.

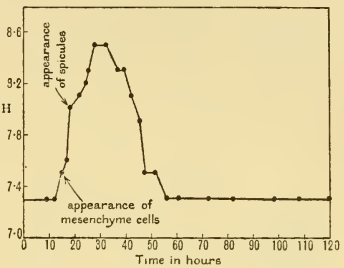

Fig. 208.

Contributory evidence showing the retention of carbon dioxide during spicule formation was provided by Vlès & Gex, who placed developing echinoderm eggs in a small chamber surrounded by a dilute indicator, and then examined the system from time to time spectrophotometrically. They obtained the figure shown in Fig. 209, from which it can be seen that during the first 4 hours the *p*H fell steadily (N.B. no sign of rhythmic change), but that between the 4th and the 6th hour there was a kink in the curve, indicating a retention of carbon dioxide, and even a slight absorption of it. This

corresponded exactly with the time of skeleton formation, as the other curve on the graph shows, relating as it does number of embryos with spicules to time. More recently, however, the micro-injection work of Rapkine & Prenant has been repeated by Chambers &

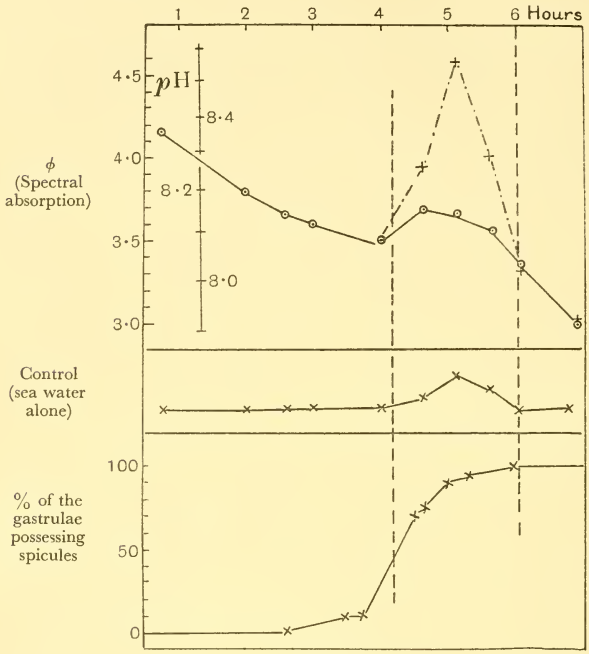


Fig. 209.

——— Experimental values —·— Corrected values

Pollack, who have not been able to find similar results. Using the blastulae of *Asterias forbesii*, *Echinarachnius parma*, and *Arbacia punctulata*, they micro-injected dissolved indicators into the blastocoele cavity, and in all cases found its *p*H to be the same as that of the external sea water. If the latter was brought down to *p*H 5 or 6, the liquid in the blastocoele cavity also gave a result of *p*H 5 or 6. Chambers & Pollack claimed that, by using very slender pipettes and

inserting them between rather than through the cells of the wall of the blastula, the *p*H of 8·4 was found for the interior fluid, and that injury to the cells surrounding it accounted for the lower hydrogen ion concentrations found by Rapkine & Prenant[1]. Chambers & Pollack concluded, therefore, that the *p*H of the liquid filling the cavity is until metamorphosis exactly the same as that of the sea water in which the embryos are placed. The regularity of the curve obtained by Rapkine & Prenant is, of course, an argument against these criticisms, but, in view of the difference of opinion, similar experiments should certainly be undertaken again.

Electrometric measurements of *p*H were begun by Vlès, Reiss & Vellinger in 1924, who made a thick suspension of eggs, freezing them solid very rapidly, pounding up the hard mass, and then placing the electrodes in contact with it as it melted. Unfortunately this procedure did not give one definite potentiometer reading, but a whole ascending curve, so that it was necessary to choose a time for taking the reading. Vlès, Reiss & Vellinger chose the moment when the thermometer indicated 0°, and calculating from that they obtained for normal *Strongylocentrotus* eggs an average value of *p*H 5·3, or for 18° 5·1. For eggs from which the jellies had been first of all removed by potassium cyanide the value was 6·3 for 18°. The three investigators explained this more alkaline figure as being due to the easier escape of carbon dioxide in the case of the dejellified eggs, though, as the whole system was one more or less homogeneous paste, it is difficult to understand this argument. In general, they concluded that the electrometric method gave results which fully confirmed their work with the microcompression colorimetric method. This was doubtless true, but it seemed to other workers that the reason was mainly because the principal source of error, i.e. cytolysis and acid production, was the same. Injury is almost certainly done to cells on freezing by the formation of ice crystals, and even if the acid production of cytolysis is in abeyance at the low temperature, it will come into play as the temperature rises, even in all probability before 0° is reached.

Vellinger, however, has continued researches in this direction, and the method has been used by the Strasburg school in the case of many types of cells other than eggs and embryos. Vellinger used a

[1] But would the cytolysis of two or three cells suffice to acidify the whole blastocoele cavity?

temperature of – 60° at which to crush the eggs, and got the same results as before, but this improvement does not make the "purée" method inherently more satisfactory. He calculated the intracellular *p*H of *Strongylocentrotus lividus* eggs to be 5·8–5·9, and of *Arbacia equituberculata* 5·0–5·2. But as Chambers & Pollack afterwards pointed out, the worst feature of this method is that the potentiometer gives a curve as the "purée" melts, and the exact point which is chosen for the reading rests on arguments no better than could be adduced for taking it at another point.

The other type of electrometric method, where electrodes are actually inserted into the egg, has not been so much used. Bodine, using a micro-electrode of his own design, requiring 0·01 c.c. of material, measured the *p*H of the egg-contents of *Fundulus heteroclitus*. The resulting mean average *p*H was 6·39, and the limits were 6·1 and 6·8. No change was to be found at fertilisation or afterwards up to 17 days' development, except that the results after fertilisation seemed to come more constant than those before. Death brought about a very acid reaction, which lowered the *p*H as far as 4·4. Placed in hydrochloric acid solutions of *p*H 4·3, the egg-contents remained quite unaffected for at least 100 minutes, but eventually changes took place inside the egg. As far as this went, the unfertilised eggs were less resistant than the fertilised ones, but there was no change in the relative resistance during subsequent development. Work on *Fundulus* was continued by Armstrong (but by micro-injection of indicators). The subchorionic space was *p*H 8·4 ( = sea water) and if the eggs were put into distilled water, descended to 5·6 in 18 hours. The pericardial cavity was still 8·4 after that time, however, as were the brain vesicles. The *p*H of the yolk was always close to 6·0.

Bodine's work was hardly a measurement of protoplasmic *p*H, in view of the highly lecithic nature of the egg of the minnow, but a number of interesting results on frog eggs were supplied by the work of Buytendijk & Woerdemann. They found that the hydrogen electrode and the quinhydrone electrode were for various reasons inapplicable to the determination of the intracellular *p*H, so they made use of an antimony electrode designed specially for the purpose. The micro-electrodes of Ettisch & Peterfi and of Taylor & Gelfan had not been suitable for *p*H determinations, but they were modified to carry Buytendijk's antimony electrode. This metal, en-

closed in a glass micropipette, proved very useful, for, as has long been known, it gives a potential difference depending regularly on the hydrogen ion concentration of the liquid with which it is in contact. Placed in one blastomere of an amphibian egg, the electrode was made to register graphically changes in *p*H. The eggs used were those of *Amblystoma*, *Triton taeniatus* and *Rana temporaria*. The main results were as follows:

| | *p*H |
|---|---|
| Ovarial eggs ... ... | 7·2 |
| Fertilised eggs ... ... | 8·5 |
| 2-cell stage ... ... ... | 8·5 |
| 4-cell stage ... ... ... | 8·05 |
| 32-cell stage ... ... | 7·9 |
| 64-cell stage ... ... | 7·9 |

As regards the intracellular *p*H, the value of 7·2 for the ovarial eggs was almost exactly the same as that of the adult blood, i.e. 7·35, a fact which recalls the osmotic pressure measurements of Backmann and his collaborators. It was not in agreement, however, with Reiss' value of 6·0 for the unfertilised frog's egg, by the micro-compression method. Newly laid eggs surrounded by solutions of *p*H 5·9 or 7·7 showed no change at all in intracellular *p*H, at any rate over a comparatively short period. Buytendijk & Woerdemann inserted their electrode into one blastomere after another in the same embryo without ever finding more than minute differences in *p*H. Cytolysis, they found, led invariably to a decrease in *p*H, obviously due to acid production, thus agreeing with our results and those of Chambers & Pollack on echinoderm eggs. The fact that the eggs which had been pierced many times with the antimony electrode still continued to divide normally indicated, they felt, that it was a very harmless instrument.

They emphasised the fact that the amphibian eggs registered a very marked change in *p*H on fertilisation, contrary to what had been found for echinoderm and teleost eggs. Their rise of 1·25 *p*H units then was in agreement with Reiss' rise of 0·4 *p*H units. But what was most noticeable about their values was that they were all distinctly higher than those of any other investigators, and while this may be due to the special material they employed, it is also very likely that of all the methods which have been used theirs does least injury to the cell, and so causes least production of acid. It would be extremely interesting to use the antimony micro-electrode on

echinoderm eggs. For later stages the following interesting values were obtained:

Table 98. Triton taeniatus.

| | | *p*H |
|---|---|---|
| Blastula ... ... ... | External cell layer | 7·6–7·8 |
| | Blastocoele liquid | 8·4–8·6 |
| Gastrula (beginning) ... | Ectoderm | 7·6–7·8 |
| Gastrula (late) ... ... | Ectoderm | 7·6 |
| | Endoderm ("Urdarm") | 8·1 |
| Neurula ... ... ... | Ectoderm cells | 6·9–7·0 |
| | Cells of neural tube | 6·8 |
| | Endoderm ("Urdarm") | 8·1 |
| | Endoderm (yolk-mass) | 6·9–7·0 |

An interesting research on the eggs of *Arbacia* was that of Vlès & Vellinger, who made spectrophotometric observations on the pigment normally contained in these cells. This involved no interference with the material at all, and only required a preliminary isolation of the pigment and a study of its properties *in vitro*. It must be remembered that the behaviour of the pigment in the cell as a *p*H indicator may only show the hydrogen ion concentration of a very limited phase, in which the indicator happens to exist. Vlès & Vellinger attempted to overcome some of these difficulties by comparing the colour change of the indicator (their "Arbacine" was probably identical with McClendon's "Echinochrome") (*a*) in alcoholic solution and (*b*) unremoved from a thawing "purée" of eggs. They showed that the colour change from orange through violet to yellow took place according to *p*H in much the same way in the two cases. By a comparison of the spectrum of the pigment when in the intact cell with the various spectra of the pigment under known conditions of *p*H, they ascertained that the former corresponded to a *p*H of about 5·5, from which they concluded that this represented the *p*H of the cytoplasmic elements or phases where the pigment was present. Spectrophotometric curves confirmed the results obtained by simply looking at the spectra, save that there was evidence of two pigments, one with two maxima and one with one. The pigment of the *Arbacia* egg is known, according to the work of Heilbrunn and many others, to be localised in certain definite elements. This means that what is probably the best possible method of studying the intracellular hydrogen ion concentration has not so far been able to give a value for the inside of a cell as a whole, or for what we roughly call protoplasm. It is to be feared that it will always be of less general

use than the micro-electrodes, because so many egg-cells are loaded with yolk and opaque substances, and so few possess a pigment which is a natural indicator.

A certain number of experiments have been made in which the effect on development of changing the *p*H of the external medium has been studied. Vlès, Dragoiu & Rose, following again their conception of "travail d'arrêt", determined the *p*H necessary for complete cessation of cleavage in echinoderm eggs. In the case of *Strongylocentrotus lividus* the percentage of eggs having accomplished the cleavage in question remained high and constant until *p*H 5·2 was reached, but between 5·2 and 4·9 it decreased by almost 100 per cent. A second paper by Dragoiu, Vlès & Rose studied the incidence of cytological abnormalities in the eggs submitted to abnormal hydrogen ion concentrations, establishing the expected result that the more abnormal the *p*H the more rapidly the abnormalities occurred. Here again, complete curves on a graph were plotted from only two points in each case. Now, although no effect on the number of cleavages in a given time had been observed between the normal *p*H (8·4) and 5·2, it was possible that a small effect had been produced. Labbe argued that this would be better shown over a longer period, so he determined the time taken to reach a definite stage in some polychaete worm embryos, obtaining figures as follows:

| | Time taken to reach a given stage in hours from fertilisation at | |
|---|---|---|
| | *p*H 8·4 (normal) | *p*H 8·1 |
| *Sabellaria alveolata* ... ... ... | 27 | 45 |
| *Halosydna gelatinosa* ... ... | 18 | 28 |
| *S. alveolata* × *H. gelatinosa* ... ... | 19 | 30 |

Evidently a comparatively small change in *p*H shows a marked effect on developmental time over a long period.

Clowes & Smith and Smith & Clowes, in a series of interesting papers, studied the effect of *p*H on various factors in development, such as the ageing of unfertilised *Arbacia*, *Asterias* and *Chaetopterus* eggs, the artificial activation of *Chaetopterus* eggs, and the development of normally fertilised *Arbacia* and *Asterias* eggs. Loeb's early work with *Arbacia* eggs had shown that addition of acid to sea water was always retarding in its action, but that small amounts of alkali exerted an accelerating action. This accelerating action was not operative before the formation of the blastula, but only between that stage and the stage of the pluteus. Excessive amounts of alkali

had, of course, an injurious effect, and the maximum was obtained when 1·75 c.c. of $N/10$ NaOH were added to 100 c.c. of sea water. He attempted to raise the eggs of *Strongylocentrotus* from fertilisation in neutral Ringer's solution without success, but found that with the addition of a little potassium hydroxide or sodium bicarbonate it was possible to do so. He concluded that a neutral or faintly alkaline solution was necessary for normal development, and subsequent experiments by Herbst and by Peter only confirmed this view. Moore,

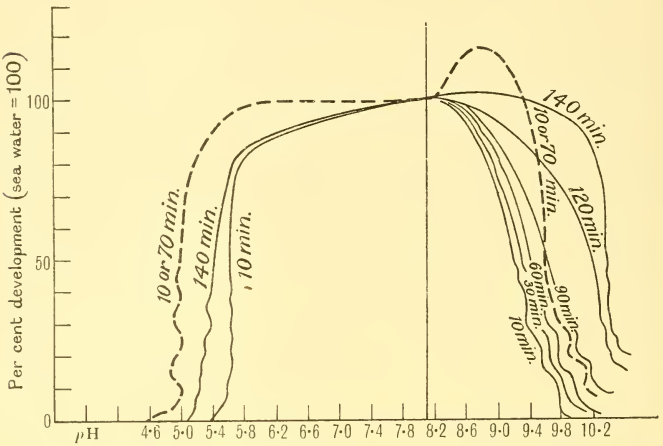


Fig. 210.

Roaf & Whitley, investigating *Echinus* eggs, found exactly the same relationships—the smallest amount of acid inhibited growth and cleavage, but alkali had first a stimulatory and then an inhibitory effect. In a later paper, Whitley could not find any evidence of the favourable effect of mildly alkaline hydrogen ion concentrations in the case of the eggs of the plaice, *Pleuronectes platessa*, but reported that a change of *p*H towards the acid side was much more fatal than one towards the alkaline side. Loeb's work with *Arbacia* eggs was repeated by Glaser, who also observed the stimulatory effect of small amounts of alkali, but emphasised that this is limited to the post-blastula stages, for the cleavage-rate at the beginning may even be

a little retarded. Medes studied the morphology of the plutei raised from solutions of varying acidity. Finally Richards observed acceleration of the early cleavage stages of the opisthobranch *Haminea virescens* in sea water to which sodium hydroxide or potassium hydroxide had been added. None of these workers had controlled the *p*H, so Smith & Clowes undertook to do so, raising these numerous isolated fragments on to a quantitative basis. Their results are shown in Fig. 210, where the percentage development (number of cleavages per egg) is shown plotted against the *p*H of the sea water, normal development at *p*H 8·15 being taken as 100. The sharp drop between *p*H 4·8 and 5·4 equates well with the results of Vlès, Dragoiu & Rose. The slight effect of hydrogen ion concentrations above normal in accelerating division is well shown on the curves; as the *p*H is raised it soon gives place to the retarding effect which brings the percentage development down to zero by the time *p*H 10 is reached. The striking thing is that the limiting hydrogen ion concentrations are characterised not by a gradual but by an abrupt inhibition of development, while between them it is essentially unimpaired. These results were afterwards repeated and confirmed by Gellhorn.

According to Smeleva and McCoy, nematode eggs are independent of external *p*H over a very wide range; an interesting fact in view of their remarkably impermeable membranes (see p. 327).

## 6·2. The *p*H of Terrestrial Eggs

As regards terrestrial eggs, those of birds have been most investigated, but we possess some figures due to Fink for the *p*H of the egg-contents of some insects, colorimetrically estimated, as follows:

| | *p*H | |
|---|---|---|
| | Soon after laying | Just before hatching |
| Colorado potato-beetle (*Leptinotarsa decemlineata*) ... | 6·8 | 6·8 |
| ,, peach-beetle (*Cotinis nitida*) ... ... ... | 6·2 | 7·1 |
| Japanese beetle (*Popillia japonica*) ... ... ... | 7·1 | 7·1 |
| Squash ladybird (*Epilachna borealis*) ... ... ... | 5·9 | 5·9 |
| Seedcorn maggot (*Hylemyia cilicrura*) ... ... ... | 5·9 | 6·6 |
| Squash bug (*Anasa tristis*) ... ... ... ... | 6·2 | 6·4 |

Thus in some cases there was no change, and in others there was a certain rise towards the alkaline side as development proceeded.

We may now take up the discussion of the total acidity and the *p*H of the various parts of the bird's egg, and the changes which it undergoes during the course of development. The classical paper on

this subject is that of Aggazzotti, but earlier workers made some observations of the kind. Thus in 1863 Davy reported that he had found in many kinds of birds' eggs that the albumen was always alkaline and the yolk acid. In 1884 Tarchanov, in the course of his work on "Tataeiweiss" already referred to (p. 272), measured the titratable acidity of the egg-whites of various eggs, obtaining the following results:

| | | Alkalinity expressed as grams potassium hydroxide in per cent. of dry weight |
|---|---|---|
| Nidicolous | Raven (fresh) | 4·9 |
| | ,, (dev. 2 days) | 1·4 |
| | ,, (still more) | 0·8 |
| | Pigeon (fresh) | 4·7 |
| | ,, (1 week) | 2·8 |
| Nidifugous | Hen (fresh) | 7·1 |
| | ,, (1 week) | 4·7 |
| | ,, (1½ weeks) | 2·7 |
| | ,, (2 weeks) | 2·3 |

Thus the titratable acidity was greater in the case of the whites of nidicolous birds than in those of nidifugous ones, and in all cases it increased as development proceeded. This has often since been confirmed. A solitary figure of Reiss' is available for the yolk and the white of an elasmobranch egg, *p*H 5·6–6·0 in the case of *Scyllium canicula*[1].

Aggazzotti's careful work on the hen's egg, published in 1913, involved many measurements, both of *p*H and titratable acidity, which are incorporated in Figs. 211 and 212. The measurements of *p*H were all made electrometrically. Taking first the graph which shows the *p*H, it can at once be seen that Davy had been quite right. The yolks of the eggs investigated by Aggazzotti had an average *p*H before incubation of 4·5, and the egg-whites one of 8·3. If the eggs were not incubated, this hydrogen ion concentration remained unaltered, as is shown on the graph by the dotted lines, and no change took place over an even longer period. If incubation occurred, however, there were marked changes in the fertile egg. The *p*H of the yolk rises steadily, attaining *p*H 6 about the 10th day of development and neutrality by about the 16th, while that of the white equally regularly falls, reaching neutrality on the 10th and *p*H 6 on the 15th day of incubation. There is thus a cross-over point when 50 per cent. of development has been completed, and after that the white is

[1] Nothing is known about the *p*H of reptile eggs but their egg-white is non-coagulable, like that of nidicolous birds (Deraniyagala).

more acid than the yolk, though neither is as far from the neutral point as at the beginning. As for the allantoic liquid, Aggazzotti did not collect many figures for it, but its *p*H seemed to follow a curve convex to the abscissa, but always near neutrality, while that of the amniotic fluid wavered around neutrality until the 11th day, after

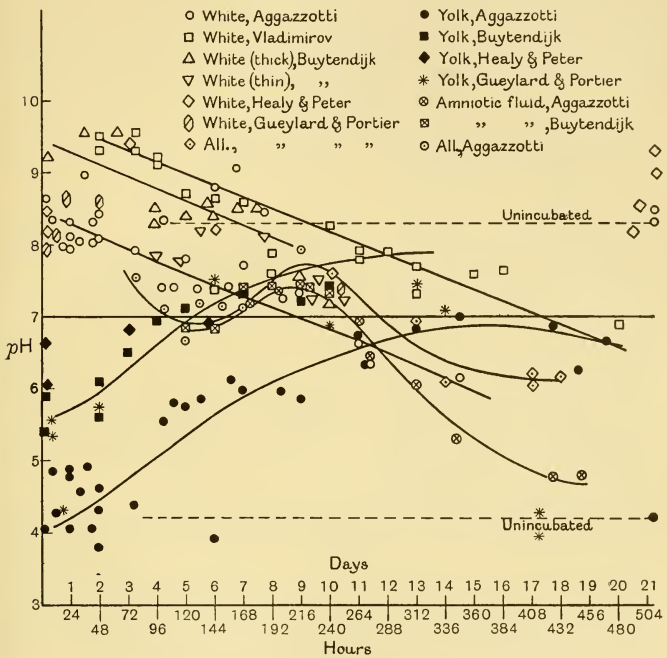


Fig. 211.

which it plunged rather suddenly into the acid region. Fig. 212, which shows the total acidity expressed in c.c. of $N/100$ $H_2SO_4$ or NaOH required to neutralise to $\alpha$-naphtholphthalein 1 c.c. of yolk or white diluted to 3 c.c. with 1 per cent. sodium chloride solution, gives a very similar picture. Just as the *p*H of the yolk rises during incubation, so there appears to be less free acid there, and just as the *p*H of the white falls, so there is more free acid

present in it. The amniotic and allantoic figures present analogous curves. Aggazzotti found that if infertile eggs were incubated there was no change at all in the *p*H of their white, but that the yolk *p*H rose just as in the fertile egg, but not so far, i.e. up to *p*H 6·2 or thereabouts, and never further. This presents an analogy with Bellini's findings on the viscosity of incubated infertile and fertile eggs, where the same change seemed to go on in the yolk of the infertile egg, but to a less extent, while the white was far less affected. It is probable that these phenomena are due to the presence of enzymes in the yolk and not in the white, a statement generally true, as the Section devoted to enzymes will show. These enzymes will naturally begin to work at the beginning of incubation irrespective of whether the egg is fertile, and will produce some of the changes associated with development, but it is reasonable to suppose that the embryo exerts a furthering influence upon their activity. This was indeed the explanation adopted by Aggazzotti.

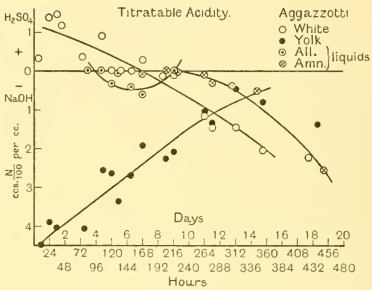

Fig. 212.

The significance of all these changes is not easy to understand. It must, for instance, be important that at the end of development the embryo is surrounded on all sides by liquids of acid reaction. The fact that the slope of the two curves (the descending one of the white and the ascending one of the yolk) is very much alike, led Aggazzotti to suggest that there was some simple relation between them, such as a transfer of hydrogen ions from the yolk to the white. But it is certain that events are more complicated than that. One very interesting fact appears, if the behaviour of the amniotic fluid in Fig. 211 is compared with that in Fig. 212, for it is then seen that, though the final *p*H of the amniotic fluid is much the same as that of the yolk at the beginning of development, their total acidities are by no means the same, the former being only half the latter. It is obvious, therefore, that the dissociation constants of the acids responsible are very different, being much greater in the case of the final amnios *p*H than in that of the initial yolk.

Since the time of Aggazzotti several workers have occupied themselves with the same problems. Healy & Peter examined the yolk and white during the early part of development with the special intention of finding what buffers were there. They obtained figures of *p*H 6·2 to 6·6 for the yolk and 8·2 to 8·4 for the white, and they confirmed the observation of Aggazzotti that no change took place over a long period if the eggs were not incubated. They estimated the alkali reserve of the white by titrating to different end points with *N*/10 HCl. Thus the following figures,

| | White | | Yolk |
|---|---|---|---|
| | Phenolphthalein c.c. *N*/10 HCl | Methyl orange c.c. *N*/10 HCl | Phenolphthalein c.c. *N*/10 NaOH |
| Incubation of 3 days | 1·4 | 15·3 | 5·2 |
| ,, 6 days | 1·7 | 14·8 | 12·9 |

indicated that the main part of the alkali reserve was in the form of sodium and potassium carbonate. Parlov confirmed Aggazzotti's results on the egg white, using the ingenious method of boring a hole in the shell and withdrawing samples each day of incubation. The *p*H fell from 9·0 to 7·0. A good deal of evidence exists that the high alkalinity of the egg-white of the new-laid egg is due to the escape of $CO_2$ from it (see Fig. 152)[1]. Thus Buckner & Martin found oviducal egg-white to be at *p*H 6·7 though after laying the usual figure of 9·0 was obtainable, and similar results were reported by Romanov & Romanov in a complete research which gave curves resembling closely those in Fig. 211. The alkalinity of the new-laid egg-white is connected with its bactericidal properties for a discussion of which see Section 19·3.

Vladimirov also measured electrically the *p*H of the white of the hen's egg during its development. His figures are shown in Fig. 211 plotted on the same graph as those of Aggazzotti. It is interesting that the fall in *p*H takes place at exactly the same rate as found by Aggazzotti, but always about one *p*H unit higher than those of the Italian worker. The still more recent figures of Buytendijk & Woerdemann agree with those of Vladimirov rather than with those of Aggazzotti, a fact which is all the more

[1] In an atmosphere containing 12 % $CO_2$ the *p*H of the egg-white remains constant at 7·8 for the first six days after laying, showing neither the usual rapid rise nor the usual slow fall (Fig. 211). And the *p*H may be varied at will by adjusting the external carbon dioxide concentration (Romanov & Romanov).

striking because their yolk figures also rise parallel with the earlier ones, only again one *p*H unit higher. All these relations are shown in Fig. 211. The cross-over between yolk and white occurs at almost the same place in all cases.

Buytendijk & Woerdemann's figures, given in Fig. 211, show a good agreement with the results of the previous observers, which is rather gratifying in view of the good technique used by them. A point of much interest is that Buytendijk & Woerdemann measured the *p*H of the less viscous and the more viscous parts of the white separately, and found that, while the *p*H of the white as a whole was falling, that of the former part of it fell more rapidly than that of the latter part. Doubtless this is due to the faster diffusion of the responsible acids into the more liquid portions. The electrometric work of Gueylard & Portier has also supplied a few figures for yolk, white and amniotic fluid, which have been incorporated in Fig. 211. Perhaps they indicate a late acidification of the allantoic as well as the amniotic fluid.

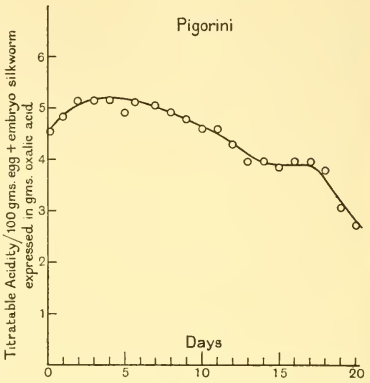

Fig. 213.

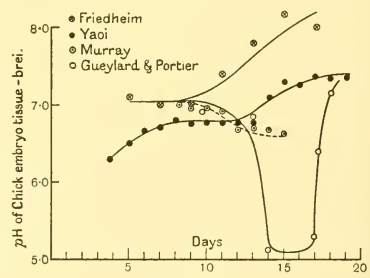

Fig. 214.

Pigorini's figures for total acidity of the silkworm egg may be mentioned here (see Fig. 213).

We may now pass to the measurement of the *p*H of the embryonic cells. For a time it was thought that results of value could be obtained by crushing the tissues in some convenient apparatus, and then estimating the *p*H of the "Pressaft" colorimetrically or electro-

metrically. Thus Gueylard & Portier employed this method, obtaining the curve shown in Fig. 214, but not putting forward any explanation for the sharp trough passed through about the 15th day. A much more complete piece of work, which involved the crushing of the cells before they were brought into contact with the indicator, was that of Murray. His figures are given beside those of Gueylard & Portier in Fig. 214, and it is unfortunate to note that, although his period was better investigated than theirs, the two do not overlap completely, so that we cannot tell whether Murray would have got the low values

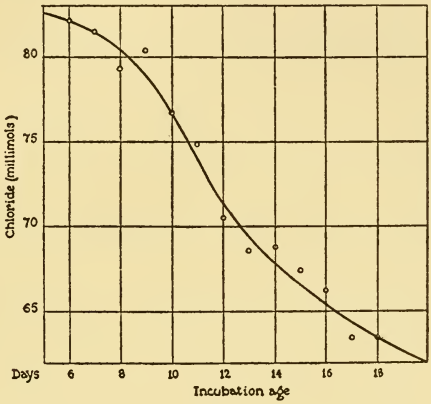


Fig. 215.

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about the 15th day if he had gone on. He found that a regular S-shaped curve (dotted in Fig. 214) would fit them, but it is unlikely that this was more than mere coincidence. As for the points of Friedheim and of Yaoi, they disagree with both the other sets.

Murray, however, estimated some other entities as well as the *p*H by crushing the cells, and it will be best to describe his results here. Fig. 215 shows the molar concentration of chlorides (determined by the Van Slyke method) in the embryonic tissues related to age, and Fig. 216 the molar concentration of total carbonic acid similarly plotted. Murray was inclined to correlate the increasing acidity of the tissues with the accumulation of the carbon dioxide of catabolism, but this, though a sufficient explanation for his own few

figures, does not cover, as it stands, those of Gueylard & Portier, still less those of Friedheim and of Yaoi. Murray called attention, however, to other factors which will obviously have to be considered with relation to the *p*H of the embryonic cells, i.e. the functional efficiency of the systems of the organism, such as the circulation, whose function it is to remove the carbon dioxide produced in

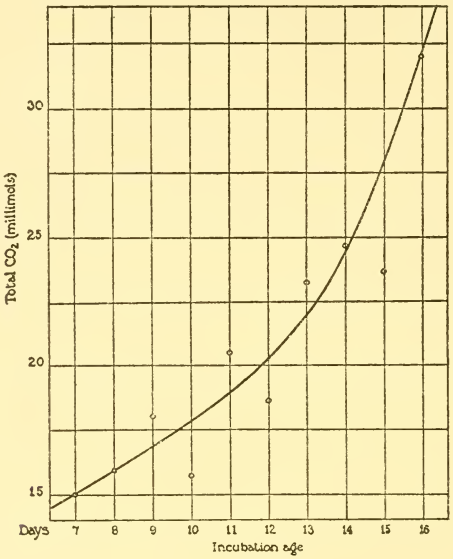



Fig. 216.

metabolism, and secondly the process of ossification, which may affect the concentration of carbonates in the tissues. Since the metabolic rate decreases with age, it alone can hardly be held responsible for the increasing acidity. Cohn & Wile showed that during the early part of development there is a marked increase in the rate and regularity of the cardiac contraction, which might explain the apparent constancy of the tissue *p*H before the 10th day. Murray related the S-shaped curve of his *p*H data to the gradient existing between the carbonates in the shell and in the bones, and

he supposed that the entry of calcium carbonate from the shell would lead to the increasing concentration of carbonate in the tissues which he found experimentally. He suggested that it might be possible to find by calculation that amount of carbonate in the functioning tissues. It was known that the concentration of protein in the embryonic body rises during incubation, so Murray suggested that, as proteins would act as anions at the *p*H found, they would replace the diminishing chloride. Unfortunately, we may be quite sure that the *p*H as found by the crushing method employed by Murray is not that of the uninjured cells, so that this calculation loses most of its force. Murray's observation, however, that the point of greatest increment on the rising protein curve came some 4 or 5 days after the point of greatest decrement on the falling chloride curve, remains quite true. "The results of our observations", said Murray, "which show that the electrolytes probably change several days before the protein, and the latter several days before the fat, lead to the conclusion that the processes of chemical differentiation are not to be described by a concept of dynamic equilibrium but rather by a notion of 'follow my leader'. The leader in this case is presumably the most rapidly permeating, reactive, and mobile molecule and tentatively we ascribe this rôle to the $CO_2$ of metabolism." What does this mean?

Parallel experiments to these were made by Woglom on the embryo of the rat, using a technique which included the fine mincing of the tissue, and the electrometric determination of the *p*H on the resulting cell-emulsion. He got values of *p*H 7·04 to 7·36, with an average at *p*H 7·14. Then Ruzicka, who did not give any details of the method he employed, but presumably worked with minced or crushed cells, obtained the following series:

Table 99.

| Frog (*Rana fusca*) | *p*H |
|---|---|
| Unfertilised eggs ... ... ... ... | 6·6 |
| Morulae ... ... ... ... ... | 6·1–6·2 |
| Gastrulae ... ... ... ... ... | 6·4 |
| First appearance of medullary plate... ... | 6·2 |
| First appearance of tail bud ... ... ... | 6·0–6·2 |
| Larva 6 mm. long ... ... ... ... | 6·4 |
| Appearance of external gills ... ... ... | 6·7 |
| Disappearance of external gills ... ... | 7·0–7·1 |
| Larva 15 mm. long ... ... ... ... | 6·8 |
| Larva 22·7 mm. long ... ... ... ... | 6·9 |
| First appearance of hind legs ... ... | 7·8 |
| Completely formed hind legs... ... ... | 7·0 |
| Immediately after metamorphosis ... ... | 7·2 |
| Sexually mature frogs ... ... ... | 7·5–7·9 |

He pointed out that a continual rise took place, a change from the acid side to the alkaline side, and suggested that it was an approach towards the isoelectric point of the tissue proteins.

Much more satisfactory was the work of Buytendijk & Woerdemann with their antimony micro-electrodes. Using them in the way described above, they obtained the following results:

Table 100.

| | Day of development of the chick embryo | | |
|---|---|---|---|
| | 4 | 7 | 13 |
| Optic vesicle ... ... ... | 6·75 | 7·3–7·4 | 7·7 |
| Brain rudiment ... ... | 6·7 | 7·0–7·4 | 7·0 |
| Myotomes ... ... ... | 7·0 | 7·5–7·6 | 7·0 |
| Liver ... ... ... ... | — | — | 7·2 |
| Heart ... ... ... ... | — | — | 6·8 |
| Stomach contents ... ... | — | — | 5·8 |
| Duodenum contents ... ... | — | — | 5·9 |
| Ileum contents ... ... | — | — | 6·8 |
| Leg muscle ... ... ... | — | — | 6·9 |

Some difference was made according to how long the estimations were done after the cessation of the circulation. On the whole, the values for the 13th day were lower than those for the 7th day, but higher than the figure obtained by Murray and Gueylard & Portier.

Another aspect of this work is the *p*H of embryonic blood. Murray made no estimations of this, but Cohn & Mirsky and Cohn, Mirsky & Porosovski afterwards made complete measurements for the chick embryo at all stages by means of the glass electrode.

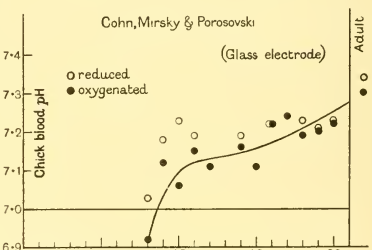


Fig. 217.

Between the 6th and the 8th days the blood was relatively acid, and from the 9th to the 15th days there was a plateau just on the alkaline side of neutrality, after which the rise towards the alkaline side was continued, to reach the adult level at the time of hatching. Experiments on the blood of the developing cat embryo gave very similar results. It is obvious that the course taken by the blood *p*H in the chick agrees closely with that of the *p*H of the yolk. There is also

agreement here with the fragmentary results of Gueylard & Portier, who found blood hydrogen ion concentrations ranging from 8·4 to 8·1 on the last 2 or 3 days of the chick's development. The few figures collected by Hajek would seem to indicate that the rise to the adult *p*H level in human blood from the acid side has attained completeness at birth.

Millet has worked on the blood and tissues of the embryonic rabbit, using the glass electrode, and Mendeléef has done similar experiments on the blood of the guinea-pig, using colorimetric

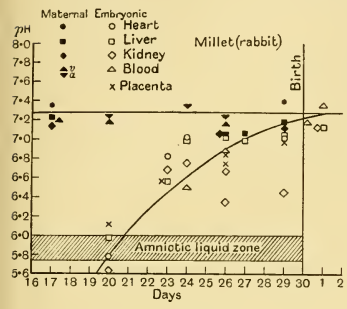



Fig. 218.

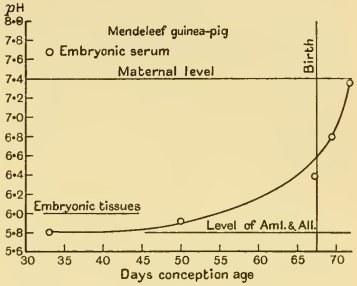



Fig. 219.

methods. From Figs. 218 and 219 it can be seen that in each case there is a passage from the acid side to slightly above neutrality, and this agrees with the work of Ruzicka; Buytendijk & Woerdemann; and Cohn & Mirsky. But there is little to show that these curves may not be due to a steady increase in the amount of blood taken for sample, rather than to a real increase in *p*H, and as yet too much emphasis must not be laid upon any of these conclusions.

## 6·3. *r*H in Embryonic Life

Closely related to the *p*H is another factor, less generally used by biologists, the *r*H. Oxidation-reduction processes, like acid-base equilibria, have an intensity as well as a capacity factor. Just as we may speak of the hydrogen ion concentration, distinguishing it from the total amount of acid or alkali present, ascertainable by a titration which mobilises the factor titrated as fast as it is used up until no more is left—so we may speak of the oxidation-reduction potential, meaning the intensity of electron transfer in the system,

as opposed to the total quantity of oxidants or reductants present. These conceptions apply, strictly speaking, only to systems which are reversible, while the living cell is a complex association of oxidation-reduction systems, some of which are probably reversible, and some of which are not. Nevertheless, the application of the conception of oxidation-reduction potential to the processes occurring in the living cell has justified itself by its results. The living cell cannot be thought of as a simple reversible system, and true equilibrium must be sharply distinguished from a steady balanced state maintained at its characteristic level by the velocities of a chain of reactions. The latter condition is what is found in the living cell, which is balanced very steadily at its characteristic level of oxidation-reduction intensity. Between the entering hydrogen acceptor, oxygen, of high $r$H, and the reducing systems such as glutathione and xanthine oxidase, of low $r$H, the cell maintains its overall $r$H closely around oxidation-reduction neutrality in ordinary aerobic conditions. These interesting problems cannot be treated here in detail, but a full account of them will be found in the series of experimental papers due to Mansfield Clark and his collaborators. The reviews of Clark; Dixon; Conant; Michaelis and Wurmser deal with the theoretical aspects of the application of $r$H to biological problems, and the review of Needham & Needham should be consulted for an account of what has actually been done in this direction. It must suffice to say here that, just as the $p$H is the negative logarithm of the hydrogen ion concentration, so the $r$H is the negative logarithm of the pressure of hydrogen gas in a platinum electrode in equilibrium with the given system, and to note that the behaviour of strong and weak acids and bases, buffers, indicators, etc., all find a counterpart in oxidation-reduction equilibria.

The earliest determinations of the $r$H of biological systems by means of $r$H indicators, i.e. dyes whose oxidation-reduction potentials at all stages of decolorisation to the leuco-bases had been accurately ascertained *in vitro*, were made by Clark. In 1925 my wife and I began a series of experiments in which these indicators (both completely oxidised and completely reduced) were micro-injected into single cells.

As regards the $r$H of the egg-cell, we reported that, in *Strongylocentrotus lividus*, *Asterias glacialis*, *Ophiura lacertosa*, *Echinocardium cordatum*, *Sabellaria alveolata*, and *Ascidia mentula*, the aerobic intracellular $r$H was always between 19 and 22. Later, Chambers,

Pollack & Cohen working with the sand-dollar egg (*Echinarachnius parma*) found a lower overall *r*H (about 12·0). We did no anaerobic experiments but the American workers got a value of 7·9 in hydrogen. No rhythmic changes occurred during segmentation nor was there any localisation of reducing power in the egg-cells. These observations were confirmed by Chambers, Pollack & Cohen. Cytolysis in amoebae produces an increased reducing power, but we were never able to see this in the case of the eggs, though we expected to do so in view of the statement made by Fauré-Fremiet that on cytolysis the reducing power for methylene blue of a given amount of *Sabellaria* eggs rises some 30 to 40 times. Chambers, Pollack & Cohen found the exact opposite to be true in the case of the sand-dollar egg; i.e. it becomes less reducing as it cytolyses. We attached much importance to the fact that no change in intracellular *r*H occurred on fertilisation, for it implied that the intensity of oxidation-reduction was not affected by that event, and the tremendous increase in oxygen-consumption associated with it must therefore be a quantitative rather than a qualitative change[1]. The same substances are combusted, we concluded, before as after fertilisation, only in less quantity. This agrees with Runnström's view that fertilisation affects the degree of dispersion of the egg-colloids, and increases the accessibility of the enzyme surfaces for their appropriate substrates. We also found the *r*H to be quite constant as far as the 8-cell stage.

Cannan investigated the oxidation-reduction potential of echinochrome, the reversible natural *r*H indicator extracted from the eggs of *Arbacia*. This pigment takes a definite place on the *r*H scale, but does not form a dissociable compound with oxygen, so that its rôle in the cell must be an activator rather than a carrier of oxygen. The pigment may therefore be an effective oxygen activator in the cell, much as the "Atmungsferment" of Warburg is supposed to be. In this case, the concentration of its reduced form present would be of great importance, and this would be determined by the oxidation-reduction potential of the cell or the phase in which the pigment is present. Arguing in this way, Cannan pointed out that only a very small change in intracellular *r*H might increase or decrease the metabolic rate or oxygen consumption by a hundredfold, so that our inability to find any change in *r*H of the cell at fertilisation—a time

[1] See further, on this subject, p. 626. Fertilisation involves "the opening of doors within the egg-cell".

when the respiratory rate abruptly and greatly increases—involved no contradiction. In the hypothetical system pictured by Cannan, very wide metabolic latitude is combined with stability of *r*H, i.e. a poised oxidation-reduction potential, and this is what actually seems to be the state of affairs in the living cell.

Cannan pointed out that in the eggs of *Arbacia* echinochrome is in the fully oxidised state, but that in those of *Echinus* it is partially reduced. As the mid-point of its *r*H titration curve at *p*H 7 is about *r*H 6, it may be concluded that the cell-granules of the eggs of *Arbacia* are more oxidising than this, while those of *Echinus esculentus* eggs are at about that figure. From the work of Vlès & Vellinger, then, we may conclude that these portions of the egg-cell have a rather acid *p*H, and from that of Cannan that they have a distinctly more reducing *r*H than the rest of the cell. Such a state of affairs may be usual in cells; thus mitochondria reduce Janus green, a very reducing dye (Needham & Needham), and Cannan found that hermidine, a natural indicator contained in plant cells, was held reduced there although active photosynthesis with oxygen production was going on, and although the micro-injection method in the hands of Rapkine & Wurmser, and other indicator work by Brooks, had demonstrated the overall *r*H of plant cells to be about 17. The living cell is without doubt extremely heterogeneous.

Micro-injection studies of intracellular *r*H were extended by Rapkine & Wurmser, who determined the average *r*H of nucleus and cytoplasm separately in the eggs of *Strongylocentrotus lividus* and *Asterias rubens*. There was absolutely no difference, both lying between *r*H 19 and 20·5. Thus the old idea of the nucleus as an "oxidation-place" (Lillie and Unna) is devoid of foundation. It remains possible, of course, that oxidations may go on to a greater extent in the nucleus than in the cytoplasm, though there is no evidence for this view; what is certain is that there is no greater intensity of oxidation-reduction in the nucleus than in the cytoplasm. They did not follow the eggs through later stages because of the disappearance of the germinal vesicle. Rapkine also studied the *r*H of the liquid filling the blastocoele cavity in echinoderms, micro-injecting indicators into it. He found it to be about 19. As he had strong reasons for supposing that free oxygen was circulating in it, it was clear that molecular oxygen was quite inactive as regards the oxidation-reduction equilibria. As the *r*H of this system was inferior to that

corresponding to the equilibrium between hydrogen and oxygen in water vapour (*r*H 28), Rapkine concluded that the activation of oxygen in the sense of Warburg no less than the mobilisation of hydrogen, in the sense of Wieland, must be important here. Much other work on plant cells, where actual bubbles of molecular oxygen may be seen traversing a protoplasm of *r*H as low as 17, led to the same conclusions, and what Clark, Cannan and Cohen call the potential of "biological" oxygen must be much lower than that of the same gas in the molecular condition.

The colorimetric *r*H measurements of Needham & Needham on marine egg-cells were afterwards confirmed by Vellinger, using a direct electrode potential method on a thawing "purée" of *Strongylocentrotus* eggs. The result so obtained, when corrected for temperature and dilution, came to *r*H 20, that of sea water to *r*H 26. The fact that our measurements and those of Vellinger corresponded so well as regards *r*H and so badly as regards *p*H might be explained by the assumption that cytolysis does not affect the *r*H, but does the *p*H, in this material.

It then occurred to Reiss & Vellinger to ask whether developing sea-urchin eggs could get their energy from hydrogen-acceptors, and not from molecular oxygen, i.e. whether the eggs could carry on their metabolism if put under anaerobic conditions. Eggs were placed in anaerobic solutions poised at different points on the *r*H scale by indicators, haemoglobin and other substances, and it was found that if the potential was 200 mv. or over, i.e. *r*H 23 or above, the cleavages would go on as usual, but if it was below that point, the number of cells dividing fell off rapidly until at 150 mv., or *r*H 22, no cells would divide. They concluded that the "*r*H d'arrêt" was always a little above the *r*H of the cell-interior. The work was shortly afterwards confirmed to some degree by Rapkine, and is very important in view of possible phases of "anaerobiosis" in embryonic life[1] (see pp. 700 and 742). The determination of the upper limit of *r*H for cell-division was subsequently made by Reiss, using potassium permanganate, sodium hypochlorite and ferricyanide. For sea-urchin eggs it was 665–720 mv. and for those of *Sabellaria* 600–700 mv.

[1] There is no contradiction between these results and those of E. B. Harvey, who studied the effects of anaerobiosis on the cleavage of echinoderm eggs. In her experiments, the cells were merely stained with methylene blue as an indicator for the disappearance of oxygen and when this was reduced, cell-division ceased. In Rapkine's experiments an excess of reducible dye was present around the cells.

In 1929 Friedheim studied the *r*H of chick embryo *Breis* from different ages, using Michaelis' mercury electrodes. There was no parallelism between *r*H and growth-promoting power (measured by the Carrel techniques), and the *Breis* seemed to be most reducing at the 13th day of development; thus:

| Age in days | *r*H | Age in days | *r*H |
|---|---|---|---|
| 5 | 6·7 | 13 | 3·9 |
| 7 | 4·9 | 15 | 5·6 |
| 9 | 4·6 | 17 | 8·6 |
| 11 | 4·3 | 19 | 10·1 |

The only investigation of the *r*H of avian yolk and white is that of Pavlov & Issakova-Keo who used a platinum electrode. The yolk of the infertile unincubated egg was always some hundred millivolts more positive than the white: the average *r*H of the former was about 24, that of the latter 28. If the eggs were kept at room temperature the E*h* of the white showed a continual tendency to become more positive, reaching after a fortnight a level of + 0·42 or *r*H 32. If incubated at 37° this positive trend occurred also in the absence of development, but if the egg was fertile the white showed on the contrary a negative trend leading to E*h* + 0·10 at 11 days, or *r*H 20. The yolk followed a corresponding downward course, leading to E*h* + 0·30 at 11 days or *r*H 18: thus always keeping at a less reducing level than the white. These facts agree well with the well-known negative trend found in cell-suspensions or bacterial cultures and indicate that the developing embryo gives off reducing substances to the rest of the egg.

## 6·4. Water-metabolism of the Avian Egg

It will be convenient to speak next of the water metabolism of embryos, a subject of considerable importance. Although the bird's egg forms one of the most complicated of the systems we have to consider, it is yet one of the best understood, and for that reason it may be taken first, the discussion then passing on to the eggs of other organisms. Another reason for dealing with the hen's egg first is that in it the embryo can be separated from the yolk at a comparatively early stage, so that separate determinations of water in embryo and food-material can be done. This is much more difficult in many other cases, such as that of the frog.

In general we may say that from the earliest point examined the chick embryo loses water relatively, although its actual content of

water in grams increases with its growth. This is shown by the curves in Fig. 220, taken from a number of investigators (Bialascewicz; Hasselbalch; Rubner; Iljin; Murray; Liebermann; Pott & Preyer), which descend very regularly, following an S-shaped course from the beginning of incubation to the end. It is unfortunate that

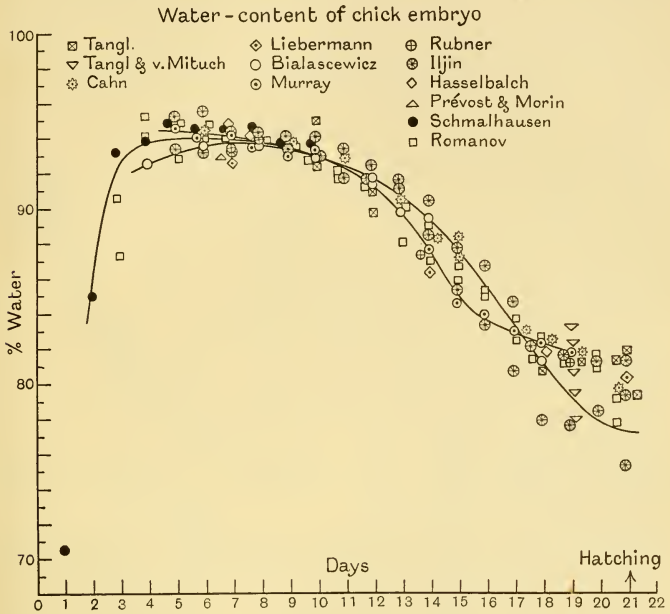



Fig. 220.

we have few data before the 5th day. A solitary determination of Bialascewicz indicates that the average value of 94 per cent., which we have at that time, may be a peak rather than a steady level and Schmalhausen, who gives a value of 70 per cent. for the second day, 85 per cent. for the third and 93 per cent. for the fourth, emphasizes this point. Further investigation is much needed here, but for the greater part of the incubation period the facts are very definite. The obvious corollary of this increasing dryness is that for every

100 gm. of water there will be more solids present at the end of development than at the beginning. The details of this concentration process were worked out for the chick embryo by Murray in the case of protein and fat, and by Needham in the case of total carbohydrate. As Fig. 221 shows, there is the gradual rise that would be expected *a priori*. It may be noticed that the only exception to this rule occurs in the case of the total carbohydrate, for from the 4th to the 8th day this value descends, showing the important part played by carbohydrate in the embryo in the very early stages. The protein uncorrected for that in the feathers has a peak on the 17th day, which is exactly what might be expected, since the feathers consist of practically dry protein, and it is about the 17th day that they form the highest percentage of the body-weight. The rise in the fat in relation to the water recalls the "lipocytic constant" and the other tissue constants suggested by Mayer & Schaeffer and Javillier & Allaire. Their behaviour during the development of the chick is very interesting (see Section 12·5). It will be important in the future to investigate further the lipoid and sterol content and concentration of the embryo, especially with a view to unravelling their influence on surface phenomena in development. The "Nachahmung" school of workers have already shown how likely it is that surface factors are one of the most important foundations of morphogenesis; it remains to establish by direct enquiry that this is really the case.

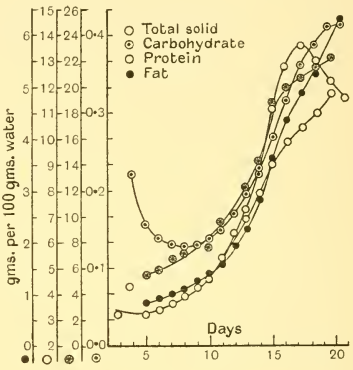


Fig. 221.

The second important factor in the water metabolism of the developing avian egg is the loss of water through the shell to the environment. At the opening of the Section on respiration a number of citations were made which showed that the loss of weight which occurs during the 3 weeks of incubation has been known for a long time, ever since the eighteenth century. Established by so many workers for the hen, Groebbels and Groebbels & Möbert have re-

cently extended it to the eggs of many other birds, mostly wild species. Their data are fragmentary, and not suitable for compression, but in all cases they found that the larger the embryo, the less was its water-content. They also found a falling weight of the whole egg during development, four examples of which are given in Fig. 222. The amount of weight lost by eggs of various birds differed, but never exceeds 30 per cent of the original weight. Table 101 gives these differences concisely. For a given bird, such as the hen, the loss is so constant that Zunz suggested frequent weighing as a guide to regulation of normal development. It is interesting to recall that other terrestrial eggs, e.g. the silkworm (Luciani & Piutti) lose water as they develop.

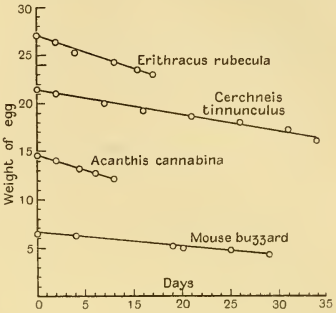


Fig. 222.

Table 101. *Loss of weight by fertile eggs during incubation.*

| Species | % | Investigator |
|---|---|---|
| Hen (*Gallus domesticus*) ... ... ... ... | 17·9 | Tangl |
| Hen ( ,, ,, ) ... ... ... ... | 14·9 | Murray |
| Hawk (*Buteo buteo*) ... ... ... ... ... | 11·5 | Groebbels & Möbert |
| Falcon (*Cerchneis tinnunculus*) ... ... ... | 23·0 | ,, ,, |
| Pheasant (*Phasianus colchicus*) ... ... ... | 16·6 | ,, ,, |
| Nightingale (*Turdus philomelos*) ... ... ... | 11·8 | ,, ,, |
| Yellow-hammer (*Emberiza citrinella sylvestris*) ... | 17·1 | ,, ,, |
| Chaffinch (*Fringilla coelebs*) ... ... ... | 26·8 | ,, ,, |
| Linnet (*Acanthis cannabina*) ... ... ... | 12·2 | ,, ,, |
| Robin (*Erithracus rubecula*) ... ... ... ... | 15·5 | ,, ,, |
| Sparrow (*Prunella modularis*) ... ... ... | 16·8 | ,, ,, |
| Warbler (*Sylvia communis*) ... ... ... ... | 17·0 | ,, ,, |
| Starling ... ... ... ... ... ... | 12·55 | Tangl |

N.B. This progressive water loss is one of the main difficulties in the way of successful *in vitro* incubation of the avian embryo, a technical problem which has not yet been solved; see Loisel (2 or 3 days), Vogelaar & Boogert (6 days), Féré; McWhorter & Whipple and S. Paton.

Iljin and Alcacid working with different incubators, some using wet air and others dry, observed a greater loss of weight in the latter, but the most complete examination of evaporation-rate is that of Murray. His immediate aim was to find the optimum conditions for

development, and to lay these down as a standard environment for future work, but his investigation of the causes of loss of weight during incubation was exhaustive. The most accurate method of finding the surface of the egg, Murray found, was expressed by the equation $S = 5{\cdot}07 \,.\, W^{\frac{2}{3}}$ (cf. Dunn & Schneider's $S = 4{\cdot}63 \times W^{\frac{2}{3}}$), but when the surface so obtained was correlated with the weight loss at standard constant conditions of temperature and external humidity, there was no significant relation.

Murray next found the weight of the shell per square centimetre, and, correlating that with the weight loss, observed a much closer correspondence. The conclusion therefore was that thickness was a more important factor in determining water loss than area, but that the influence of both these factors was negligibly small. Probably an egg with a heavy shell which has small rarefied areas may lose more weight in a given time than one which has a lighter shell of uniform thickness. Eggs in which minute cracks were made supported this view by losing as much as 100 per cent. more weight each day than the average normal eggs. Murray weighed the shells taken from eggs of different incubation times, and comparing his results with those of Carpiaux; Tangl; and Plimmer & Lowndes, who had made shell weighings in connection with calcium analyses, he concluded that on the average 0·01 gm. of shell-substance are lost to the interior of the egg for every increase in embryo weight of 1·0 gm.

He next studied the loss of water by the eggs at different positions of the two main variables, i.e. temperature and humidity. Fig. 223, taken from his paper, shows the loss in weight of White Leghorn hen's eggs during the incubation period in standard conditions: $T = 38{\cdot}8 \pm 4°$, humidity $67{\cdot}5 \pm 2{\cdot}5$ per cent., continuous flow of warm air, eggs turned once a day. There was no perceptible difference between fertile and infertile eggs until the 16th day was reached, after which the fertile ones tended to lose more weight than the infertile ones. In Fig. 224 is shown the effect of humidity; evidently the most important factor of all. At 100 per cent. humidity the egg loses no weight. With regard to the fact that the fertile eggs lost constantly rather more weight during the last week of incubation than the infertile ones (found also by Bywaters & Roue and Romanov), Murray pointed out that three possibilities presented themselves to account for this: (1) that the expired carbon dioxide was greater by weight than the oxygen absorbed during the

same period, (2) that some other gaseous product was eliminated, or (3) that there was an increased evaporation of water. Neither of the first two of these theories is very agreeable with the facts, for, if (1) were true, the predominant substance combusted during the last week would have to be protein, and we know almost certainly that this is not the case, while all that is known about the metabolism of the egg is against the second possibility (see the Section on respira-

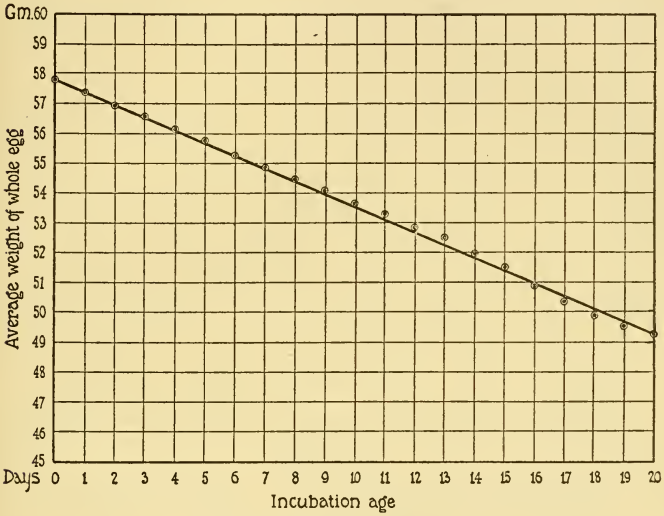
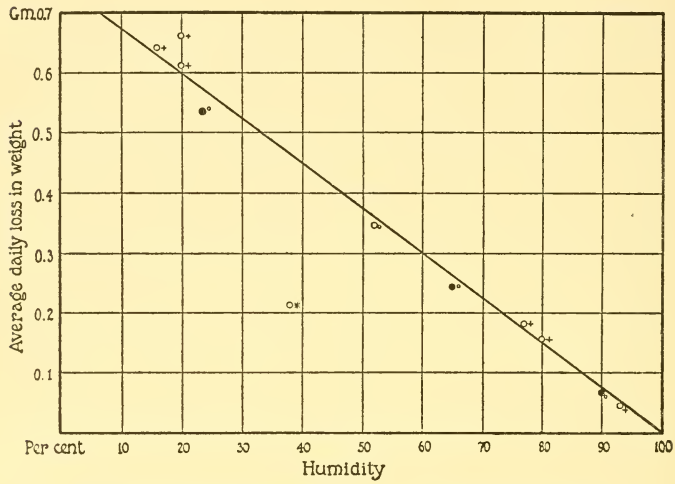



Fig. 223.

tion). The third alternative must then be the correct one, and, although it is not easy to see how the presence of the developing embryo could increase the water elimination, it must be remembered that the embryo is producing heat, and that evaporation would be accelerated thereby. Probably the circulating blood in the allantoic membranes, said Murray, is a more efficient evaporating mechanism than the undifferentiated albumen.

The dependence of water loss on external humidity demonstrated by Murray shows that birds' eggs have no power of controlling the amount of water they lose. This conclusion had been previously

arrived at by Aggazzotti, who studied the water loss from hen's eggs at sea level, and at the high-altitude research station at Col d'Olen. He found that at a height of 250 metres above sea level the eggs lost more water each day than at Turin (sea level), exactly contrary to what took place in the case of the adult animals, which lost more water each day at Turin than at Col d'Olen. There was evidently




● Whole series of experiments. + Eggs kept above 38·8°.
○ Eggs measured every two days. o Eggs kept below 38·8°.
* Eggs kept at room temperature.

Fig. 224.

no regulatory mechanism in the hen's egg, and Aggazzotti compared the initial unprotected state of the egg as regards water loss with the assumption of homoiothermicity which takes place during development.

Interesting experiments on the evaporation rate of hen's eggs have been carried out by Dunn. Individual eggs of White Leghorn breed gave evidence of great variations in the rate at which they lost water. The data for the effect of incubation, presence of living embryo, etc.,

were in general agreement with the later independent results of Murray. Dunn found that eggs were two or three times as variable in their evaporation-rate as in their original fresh weight. If, then, the rate at which an egg loses weight is regarded as an index of the permeability or porosity of its envelopes, then the shells of eggs must be more variable than the sizes of the eggs themselves. This was exactly what had been found by Curtis for the weights of the shell, the coefficients of variation of shell-weight/egg-weight being 10·43/6·36. In sum, loss of weight, said Dunn, was to be regarded as an individual character, like weight, length, breadth, shell-weight, yolk-weight, etc. In his second paper, he studied the relation of egg-size to weight loss, and concluded that larger eggs, though they lose more actual weight, lose an appreciably smaller proportion of their weight than smaller eggs. The larger eggs can apparently better conserve their moisture-content, but this is due wholly to their relative surface. The shells of the larger egg, however, were somewhat less porous, for they lost less weight per unit of surface area. This shell-difference was the subject of the third paper. Analyses made on the shells of eggs which had shown high and low rates of evaporation respectively revealed no difference except in the shell-weight, thus:

| Rate of loss | Egg-weight (gm.) | Shell-weight (gm.) | Shell-weight (%) | Calcium oxide (%) | Magnesium oxide (%) |
|---|---|---|---|---|---|
| High... ... | 65·22 | 5·399 | 8·28 | 52·01 | 1·52 |
| Low ... ... | 55·38 | 5·258 | 9·49 | 52·44 | 1·52 |

But, on the other hand, the rate of evaporation was correlated very closely with the number of pores (see Rizzo, p. 722) in the shell, and this was indeed quantitatively much the most important factor. Later Dunn investigated the relations between evaporation-rate and hatchability. He found that under constant conditions the rate at which a normal fertile egg loses weight in the first 7 days of incubation played little or no part in determining the subsequent fate of the embryo contained in it. Nor was the weight loss during the 2nd week correlated in any way with the hatchability. On the other hand Romanov found that the optimum hatch occurs at 60 per cent. humidity. He weighed a large number of embryos from eggs kept at 40 and 80 per cent. humidity, and concluded that the latter condition gave slightly heavier embryos than normal although their percentage dry weight was less than usual. At high humidity the percentage ash

was high in the embryonic body. Too high humidity gave, he found, a worse mortality than too low.

We have seen, then, that the egg as a whole is continually losing water at a constant rate, and that the embryo is continually gaining it at an ever-decreasing rate, so that it becomes less and less wet as it develops. The third cardinal fact in the water metabolism of the hen's egg is that the yolk is gaining water at the expense of the white. This process has already been alluded to in the Section on

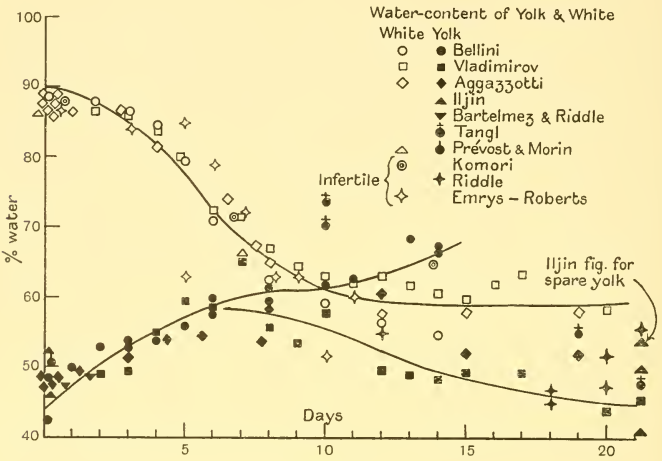



Fig. 225.

biophysical phenomena, where the viscosity and the osmotic pressure of the yolk and the white necessitated its mention. There is reason, in fact, for believing that the yolk absorbs water from the white from the moment at which they first come into contact, i.e. in the oviduct, but it is probable that the mechanism by which this is done differs as time elapses, and as the embryo grows. The actual determinations of the water-content of yolk and white are assembled in Fig. 225, from which it can be seen that the results of all investigators from Prévost & Morin onwards agree well together. A little uncertainty, however, exists with regard to the course taken by the yolk after the mid-point of development, for Bellini's figures would

make it seem as if its water-content remains unaltered after that time, whereas the results of other workers show a descent to about the same value as in the unincubated egg. It is probable that there is a descent, for the dense and viscous condition of the yolk at hatching is familiar[1]. The course of affairs, then, may be summed up by saying that for the first 10 days a flow of water passes from white to yolk. After that time the water-content of the white remains constant, but, owing to its now very small size, becomes quite unimportant in absolute reckoning, while that of the yolk declines again to its original figure. Carini, without giving any figures, stated that the yolk-volume increased at the expense of that of the white, which is in agreement with the rest of our knowledge about it. He also found that the digestibility of egg-white by pepsin decreased as incubation proceeded, and he put this down to its decreasing water-content.

What is the mechanism of this passage of water into the yolk? Greenlee supposed that the greater concentration of osmotically active substances in the yolk would amply account for it, and that the transference of water was entirely due to osmotic causes. He found that the rate of loss of water by infertile whites followed an extremely regular course, rising with the temperature, and falling with the time. He constructed curves for this, and was able to express the whole process by an equation on the basis of which the moisture content of the yolk and white of an infertile egg at any given time after laying could be predicted for a given temperature and a given initial state. But the evidence in favour of the water-current of infertile eggs being purely osmotic in nature is not satisfactory (see p. 816).

In the developing egg, however, matters are certainly more complicated. Vladimirov criticised the older viewpoint, showing by means of a simple calculation that the observed osmotic pressures would not account for the movements of the water. He suggested

[1] The question of the "bound" water in the egg is one of much interest. Bound water may be defined as that part of the water in which added solutes will not dissolve. Zawadzki showed that the free water in the yolk is probably identical with the intermicellar liquid (the ultrafiltrate) of Bialascewicz (see p. 361). By adding known amounts of sucrose and urea Hill found that 97 % of the water in the egg-white was free, and 85 % of that in the yolk. One egg-yolk would thus contain (besides 7·95 gms. solid) 6·05 gms. of free and 1·05 gms. of bound water; one egg-white would contain (besides 6·39 gms. solid) 22·9 gms. of free and 0·71 gms. of bound water. Nothing is known of the variations which may occur in these factors during the development of the embryo, or of the bound water in the embryo itself.

that an important factor besides osmotic pressure was the amount of water held back by the protein of the egg-white, the "Aufquellungswasser". In protein solutions of above 40 per cent. the intensity of this force can reach several atmospheres. In order to penetrate further into these complex relations, Vladimirov measured the electrical conductivity of the egg-white during development, as an index of what was happening to the electrolytes. The results are shown in Fig. 226. If the eggs were infertile and not incubated, there was no change, the electrical conductivity remaining in the close neighbourhood of $7{\cdot}6 \cdot 10^3$ Kohlrausch units (1 K. unit = the conductivity of a substance of which a column

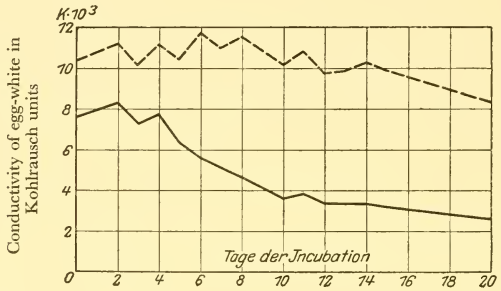



Fig. 226.

1 c.cm. long and 1 sq. cm. in area opposes a resistance of 1 ohm). If the eggs were infertile and yet were incubated, there were only small changes, which led to an increased conductivity, but if normal development went on, there was a marked downward trend, the conductivity reaching a minimum of $2{\cdot}53 \cdot 10^3$ on the 20th day. These results are in agreement with those of Bellini (see above, p. 830), and would have indicated a definite decrease in the electrolytes present in the white had not Vladimirov made a correction for the large amount of protein present. This was based on theoretical grounds (see the original paper) and showed, as appears from the dotted line in Fig. 226, that the electrolyte-content really remains constant, i.e. the absorption of electrolytes moves parallel with the absorption of water, and can therefore play little or no part in the mechanism governing the latter phenomenon. Osmotic pressure

measurements, which have already been referred to above, led Vladimirov to the same conclusion. After the 6th day, there is little or no change in the osmotic pressure of the egg-white. Finally, he examined the *p*H of the egg-white at different stages, with the results which have already been described (see Fig. 211). His *p*H measurements were done after removal of carbon dioxide by bubbling of hydrogen and subjection to a vacuum, so that the acid responsible for the decreasing *p*H of the white must have been a fixed one, and could not have been simply $CO_2$. Thus the tendency towards the acid side of neutrality, brought about by the presence of this unknown acid in increasing concentration, would lead, Vladimirov argued, to a closer approach to the isoelectric point of the egg-white proteins (between *p*H 5·0 and 6·0), and therefore to a loss of the power they possess of holding water, their "Quellungsfähigkeit". In this way the current of water yolkwards may be better explained. It should be noted that all the constituents of the white pass into the yolk or the embryo, but that when they do so at a much greater rate than the average we speak of a yolkwards current. Thus the salts also pass into the yolk, but not faster than the protein of the white is itself absorbed by the embryo.

Bartelmez & Riddle published some data which add to our knowledge of the current of water. The following table is striking:

Table 102.

| | % water in yolk | | | | |
|---|---|---|---|---|---|
| | Ovary | Oviduct | Fresh-laid | Incubated 24 hours | Incubated 4–9 days |
| *Gallus domesticus* ... ... | 46·17 | 46·79 | 47·59 | 48·40 | — |
| *Turtur orientalis* ... ... | — | 55·21 | 55·83 | 56·94 | 58·80 |
| *Streptopelia alba* ... ... | — | — | 55·80 | 57·11 | 58·68 |
| *Columba oenas* ... ... | — | — | — | 56·80 | 59·93 |
| *Columba livia domesticus* ... | — | — | 54·71 | 57·17 | — |
| *Columba guinea* ... ... | — | — | 54·97 | 55·61 | 60·15 |

They pointed out that, as far as the fowl was concerned, the time of most rapid absorption of water by the yolk was before laying, and they suggested that probably the liquid which fills the sub-germinal cavity in the bird's egg was derived from this source. Digestion of the upper part of the latebra would play a part in the production of the sub-germinal cavity, as was first made likely by Patterson, and it is a remarkable coincidence, if no more, that the cavity is formed during the period of most rapid water absorption. Bartelmez

& Riddle found that the fluid filling the subgerminal cavity in the pigeon's egg would hardly form a coagulum when heated, and evidently contained only a little protein.

Closely associated with the water metabolism of the egg is the origin of the amniotic and allantoic liquids. Kamei has studied their increase in volume (see Fig. 227). The allantoic fluid shows at first a fairly regular rise and by the middle of development reaches 6 c.c., after which it probably falls. These 6 c.c. represent about 15 per cent. of all the water in the egg, i.e. that percentage has been required to assist in the excretion of from 7 mgm. (Fiske & Boyden) to $3\frac{1}{2}$ mgm. (Needham) of uric acid (see p. 1092). The reabsorption of water from the allantois must begin very soon after the mid-point of development, for by the 12th day its uric acid content is increasing while its volume is remaining steady or diminishing. The amniotic liquid, on the other hand, reaches what is practically its maximum by the 10th day and does not fall until the final desiccation of the egg before hatching begins.

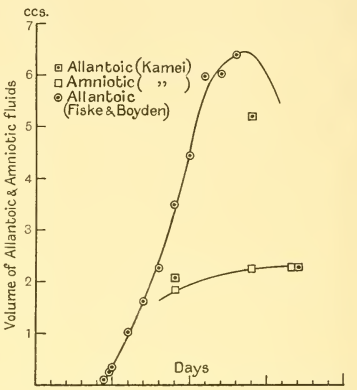



Fig. 227.

The specific gravity of the amniotic liquid rises to a maximum on the 14th day but that of the allantoic liquid rises throughout development, as follows:

| Day | Amniotic | Allantoic |
|---|---|---|
| 9 | 1·0062 | 1·0070 |
| 14 | 1·0630 | 1·0147 |
| 17 | 1·0400 | 1·0205 |

The eggs of reptiles, especially turtles, contain white[1], and Agassiz gave a description of the histology of their yolk after laying, which has been interpreted by Bartelmez & Riddle as indicating

[1] Deraniyagala describes the white as at first viscid and later quite mobile and limpid. This is precisely the opposite to what happens in avian eggs (see Figs. 207 and 215) but would be expected from the considerable water-intake (see p. 898, Table 105).

an absorption of water by the yolk in that case also. Agassiz also noted a liquefaction of the albumen in the fowl's egg immediately over the germinal disc, corresponding to the liquefaction underneath it.

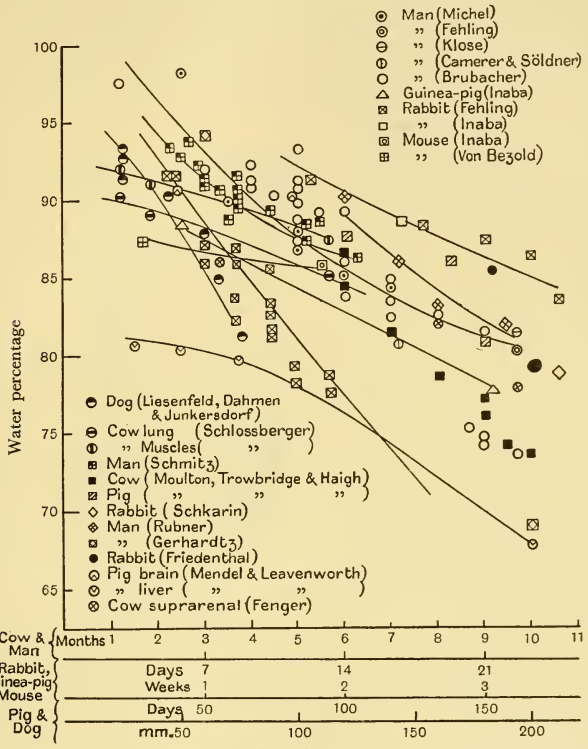



Fig. 228.

## 6·5. Water-content and Growth-rate

In birds, then, the general rule seems to be that the water-content of the embryos is higher the younger they are. In many other cases, the same relationship appears. Especially in the case of mammalian embryos is this true; thus Fig. 228, where a good deal of the data is collected, shows a continual fall for man (Fehling; Michel; and

Brubacher), for the guinea-pig (Inaba), for the rabbit (Fehling), the mouse (von Bezold and Inaba), and the cow (Moulton, Trowbridge & Haigh). Moreover, the process is continued after birth, as the complete figures of Hatai for the rat, and of Thomas and of Weigert for the cat and dog clearly show. On the basis of these facts, several authors have concluded that a decreasing water-content is a universal accompaniment of growth, and the more rapidly the latter takes place the more rapidly does the drying up of the tissues go on. Cramer found that the water-content of neoplasms was always higher by at least 5 per cent. than that of normal tissues, and that it varied directly with the growth-rate of the particular neoplasms, i.e. those growing the fastest as measured by mitotic index had the most water (see Fig. 229). Cramer concluded that a high water-content was intimately associated with a high growth-rate. Then Ruzicka later was much impressed by these facts, and enunciated a "law of protoplasmic hysteresis", while Rocasolano and Le-Breton & Schaeffer pointed out its relations with the paraplasmatisation of the tissues with age (see p. 747). Ruzicka regarded the ageing process as a continual tending of protoplasm towards stability, solidity, insolubility and dryness. Some curious digestion experiments which he made are relevant here. The time required to dissolve embryonic tissues by trypsin under standard conditions he found to vary as follows:

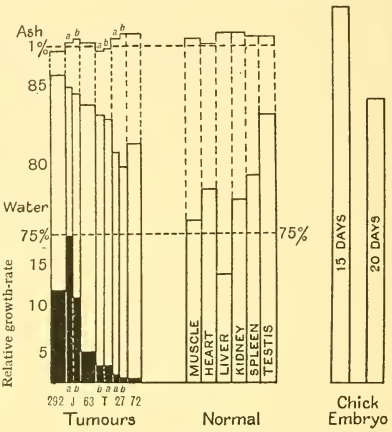



Fig. 229.

292, J, 63, T, 27 & 72 are strains and *a* and *b* generations of strains.

| | | Hours |
|---|---|---|
| Frog. | Blastulae ... ... ... ... | 3– 6 |
| | Embryos with tail buds ... ... | 26–32 |
| | Embryos with external gills ... | 43–53 |
| | Tadpoles 19 mm. long ... ... | 71–80 |

In all cases there was an insoluble residue which increased in amount per gram of original material with age. Ruzicka also showed that the

older an individual the more easily can flocculation of the proteins of its tissue juice be brought about. Thus the "Pressaft" of frog's eggs required 2·66 c.c. of 96 per cent. alcohol for flocculation, while that of 10-year old frogs required only 0·9 c.c. This phenomenon was related, he thought, to the isoelectric point of the cell-protein. Coherence, degree of condensation, hysteresis (passage from sol state to gel state) with many other concepts all intimately associated with

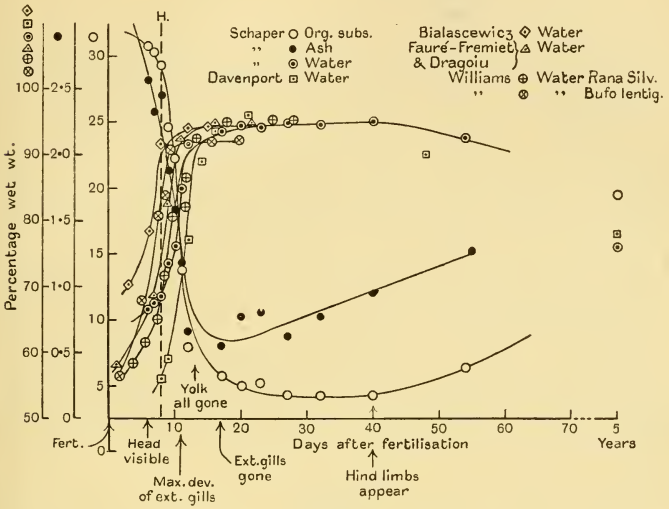



Fig. 230.

decreasing water-content, form the components of Ruzicka's general theory.

In spite of this agreement, however, some facts had been known for a long time which seemed to oppose the introduction of a general law of decreasing water-content. The work of Davenport; Schaper; Fauré-Fremiet & Dragoiu; and Bialascewicz on the early stages of amphibian development seems at first sight to be in contradiction with it, for, as Fig. 230 shows, the water-content rises steadily until about a week after hatching. Kaufmann, again, showed that salamander larvae cut out of the uterus took up much moisture when

put into distilled water. Here, however, it must be remembered that the embryo cannot be separated from the yolk-sac, so it is the water-content of embryo plus yolk that is being measured. And where the yolk is allowed for, as in v. Bezold's work on *Bombinator igneus*, the water-content follows the usual rule and decreases, thus:

| | % |
|---|---|
| Embryo ... ... ... | 90·6 |
| Hatched larva ... ... | 86·7 |
| Tadpole ... ... ... | 81·2 |
| Adult ... ... ... | 77·3 |

On the other hand in the case of fish eggs it is possible to separate the embryo from the yolk from a comparatively early period onwards, and for the trout, where this has been done, the water-content of the embryonic body does not seem to change at all. Fig. 232, constructed from the figures of Kronfeld & Scheminzki and of Gray shows this very definitely, and it would seem that the water-content of the trout embryo never varies much from 85 per cent. though, of course, it may be higher in the earlier period which was not investigated. From the work of Fauré-Fremiet on the egg of *Sabellaria alveolata* it is not possible to learn much, for the yolk was never separated from the embryo, and we cannot say therefore what was the significance of the loss of 5 per cent. in dry weight. Teissier's study of the development of the medusa *Chrysaora hyoscella*, however, demonstrated that, although the adult medusae have a great deal of water in their bodies, the early stages have much less. Thus he obtained the following results:

Table 103.

| Age of medusae | % water | Water per gram dry weight | Ash | Organic substance | Phosphorus |
|---|---|---|---|---|---|
| Planulae <1 day | 64·9 | 1·84 | — | — | — |
| Planulae 1–2 days | 66·3 | 1·97 | 2·3 | 31·4 | 0·33 |
| Planulae 2 days | 67·5 | 2·07 | — | — | — |
| Planulae 3–4 days | 71·0 | 2·45 | 3·2 | 25·8 | 0·27 |
| Schyphistomes | 94·5 | 17·00 | — | — | — |
| Adult medusae | 96·3 | 26·00 | — | — | — |

But it might perhaps reasonably be argued that the water-content of medusae is so unusual that the ordinary relations would not be expected. Histological arguments supported the view that, while the early increase in water-content was due to intracellular swelling, the later increase was intercellular, due to the mesoglia.

Wetzel's comparative work introduces further complications. He analysed the eggs of a number of different kinds of animals, and then adopted the unsatisfactory expedient of comparing the results with figures for adults of the same kind, taken from other investigations. Thus he drew up the following table:

Table 104.

| | Water-content % | |
|---|---|---|
| | Egg (Wetzel) | Adult (Sempolovski) |
| Sea-urchin (*Strongylocentrotus lividus*) ... | 77·9 | — |
| Starfish (*Asterias glacialis*) ... ... ... | — | 67·36 |
| Spider-crab (*Maia squinada*) ... ... ... | 56·4 | — |
| Cray-fish (*Astacus fluviatilis*) ... ... ... | — | 77·11 |
| Crab (*Cancer pagurus*) ... ... ... ... | — | 62·64 |
| Dogfish (*Scyllium canicula*) ... ... ... | 43·6 | — |
| Ray (*Raia radiata*) ... ... ... ... | — | 80·67 |

But we cannot conclude from this table that the echinoderms get drier as they develop or that the crustacea and elasmobranchs get wetter, for we learn nothing from it about what is the real centre of interest, the embryonic body itself. According to Ephrussi & Rapkine the sea-urchin's egg absorbs water from the sea.

| | | Wet weight | Dry weight |
|---|---|---|---|
| Change: unfertilised egg as unity | 12 hours ... | +8·8 | +9·0 |
| | 40 hours ... | −1·4 | +5·6 |

Just as there is still much uncertainty about the behaviour of water in the entire embryonic organism, so the data we have for its separate tissues are rather complicated, if not contradictory. For muscle tissue, Jacubovitsch stated in 1893 that the water-content decreased with the age of the embryo; his figures are shown plotted in Fig. 231 together with those of Mendel & Leavenworth on the brain and liver of the pig. Bischov, again, had found the figures:

| | Whole man | Muscle |
|---|---|---|
| Newborn ... ... | 66·4 | 81·7 |
| 33 years ... ... | 58·5 | 75·67 |

Then Fauré-Fremiet & Dragoiu, in their work on the embryonic lung in the sheep, obtained a curve which fell from the 7th to the 14th week, after which the determinations became impossible owing to the swallowed amniotic liquid. As regards nervous tissue, Glaser's results on the brains and spinal cords of *Amblystoma* indicated a constancy in water-content; thus the just-hatched *Amblystoma* brain

had 82·8 per cent. and the cord 79·0 per cent., while Donaldson found for *Rana* adult brain 84·9 per cent. and cord 80·5 per cent. Rosenheim, on the other hand, found in human brain 90·29 per cent. for the 36 weeks' foetus and 85·8 per cent. for the infant shortly after birth, and a trend in the same direction was found by Gundobin and by Koch, who obtained the following figures:

| | % |
|---|---|
| Brain of foetus of pig 50 mm. long ... | 90·78 |
| Brain of foetus of pig 100 mm. long ... | 91·01 |
| Brain of newly-born albino rat ... ... | 89·58 |
| Brain of adult albino rat ... ... | 78·10 |

It is safe to say, then, that, though in certain cases the water-content of embryonic tissues seems to remain unchanged with age, there are a few instances of a rise, and the majority of experiments show that it falls. The explanation of this fall is difficult, though, as we have observed, some investigators have seen in it perhaps the most fundamental attribute of the ageing-process. A point which does not seem to have been noticed so far is that the high water-content in the early stages may be related to the importance of primitive connective tissue and its contained lymph. I have already drawn attention to the primitive undifferentiated connective tissue, first discovered by von Szily, in connection with the initially high concentration of glucose in combination with protein[1], and it will again be mentioned in connection with the relatively high silicon content of young embryos. But what is important here is the fact that connective tissue has always a high water-content, and this might explain the phenomenon now under discussion. Thus Skelton found that bleeding in mammals produced a flow of water from the tissues into the blood, and by appropriate experiments he was able to ascertain what per-

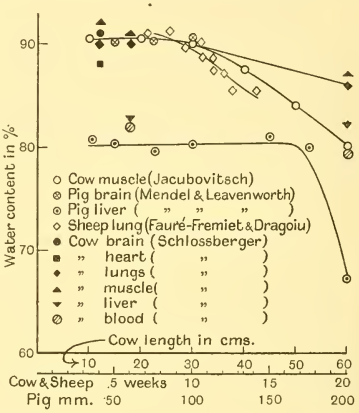



Fig. 231.

[1] See p. 566.

centage had been contributed by each of the tissues. His figures were striking.

| | % of the total water contributed to the blood |
|---|---|
| Muscles ... ... ... | 10·0 |
| Liver ... ... ... ... | 8·0 |
| Intestines ... ... ... | 2·0 |
| Spleen ... ... ... ... | 0·3 |
| Connective tissue ... ... | 78·0 |

There is thus the possibility that the decreasing water-content of embryos may be simply an index of the decreasing amount of primitive connective tissue. From the point of view of paraplasmatisation or degree of diminution of active protoplasm, it would be desirable to test the truth of this view in some way. The original suggestion of connective tissue and lymph as playing this rôle is due to Schlossberger. From the experiments of Wiener we know that, in the human foetus, the lymph circulation is active from an early date. Foetal lymph has been analysed by Raske (see Section 23·7).

## 6·6. Water-absorption and the Evolution of the Terrestrial Egg

We may now return to the curve for the rising water-content of the amphibian larva (embryo plus yolk) established by so many workers and plotted in Fig. 230. It has usually been regarded as a fundamental property of this phase of embryonic growth, but Arager contests such a view. Arager removed the jellies from frog neurulae, and put them to develop in a chamber the humidity of which could be controlled. In this way it was possible to produce larvae of very much less water-content than normal, and Arager noted that, in such larvae, the histological differentiation of the tissues was normal, although the morphological relations of the organs might be considerably upset.

But leaving this difficult question, we shall find it advantageous to study the results obtained by Kronfeld & Scheminzki and by Gray on the trout embryo. As has before been mentioned, this can be separated from its yolk at an early period. In general, it resembles the amphibia, for a preliminary period inside the egg-membranes is succeeded by a free-swimming period in which the fish lives on the yolk in its yolk-sac, and this in turn by a period in which ordinary ingestion of food by the mouth is begun if there is anything present to eat. As Fig. 232 shows, the water-content of the trout embryo is

constant at about 85 per cent. during the last third of the first period and the whole of the second period. The water-content of the yolk is again uniformly low, about 60 per cent., and the fact that the water-content of the whole system, embryo plus yolk, rises, is evidently due to the fact that the embryo is growing all the time in size relatively to the yolk which is diminishing in size. Where the water of the embryo comes from is a matter which we may shelve for the moment. Now there is a strong probability that the type of graph shown in Fig. 232 for the trout may also turn out to be applicable to the frog. Fig. 233 taken from Gray's paper, shows the decreasing

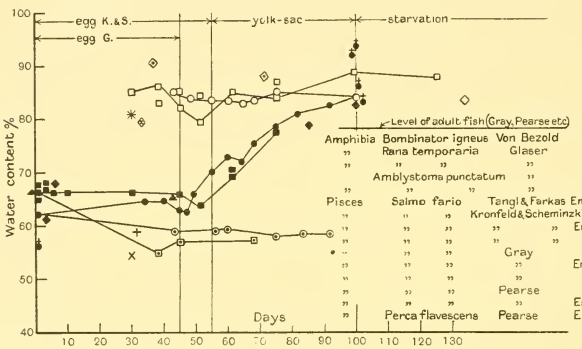



Fig. 232.

dry weight content of the larva (embryo plus yolk) in the frog and the trout, so that, as far as that goes, they behave similarly. It was said above that the frog larva is not capable of being separated into its embryonic and yolk components, but this is not strictly true, for a few careful dissections made by Glaser are available to throw light on the matter. Glaser separated the embryos of *Rana temporaria* into two parts, one predominantly consisting of yolk, the other of "nervous tissue". Water estimations on these gave the figure of 54·2 per cent. for the yolk and 80·1 per cent. for the embryonic body; these are plotted on Fig. 232, from which it can be seen that the relations are just like those found by the workers on the trout. It is, therefore, probably right to conclude that what happens both in the frog and the trout is a constancy in water-content of the em-

bryonic body, and a rise in the water-content of the embryo-plus-yolk system.

The obvious question now arises, what is the origin of the water which helps to build up the tissues of the frog and the trout embryo. Gray's work on the brown trout, *Salmo fario*, provides a graph showing the relative amount of embryo and yolk in the different stages; this is given in Fig. 234. The wet weight of the embryo in percentage of

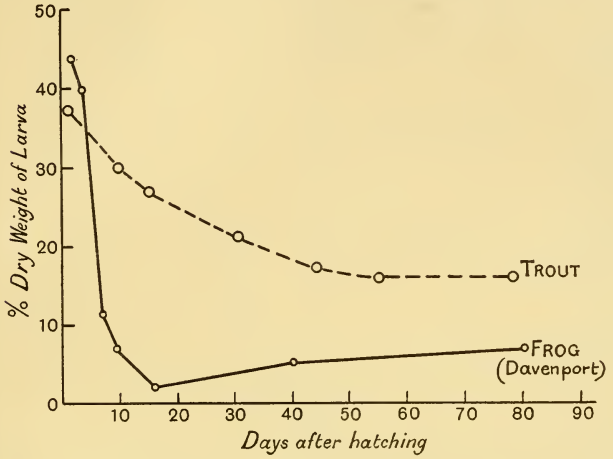



Diagram illustrating change in water content of Larvae

Fig. 233.

the wet weight of the system embryo plus yolk is plotted agains the time, and the resulting curve is S-shaped. It contributes to the view that the rising water-content of the whole system (shown in Fig. 235, which is also roughly S-shaped), is due entirely to the increasing preponderance of embryonic tissues, which maintain a constant proportion of water within themselves. "In other words", as Gray says, "as yolk is converted into embryo, water is added from the external environment and the yolk may be regarded as more or less desiccated nutriment. We can therefore conclude that the concepts of 'passive' and 'active' growth (Davenport) have no

foundation in fact." Now the initial weight of wet yolk in a newly fertilised trout's egg is approximately 100 mgm., and, as 41 mgm. of dry yolk is present, an amount of fish can be built up with these materials corresponding to 256 mgm., for the water-content of the

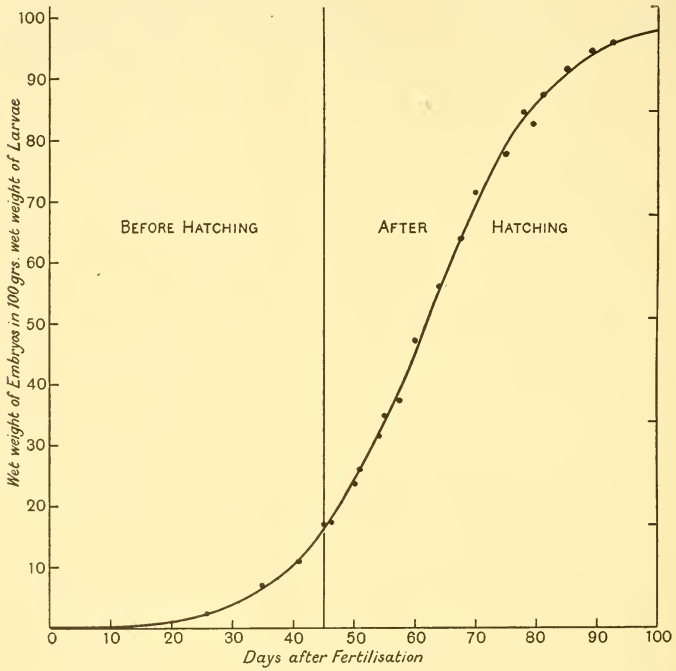



Fig. 234.

final product may be taken at 84 per cent. Thus there is enough dry material in the fertilised egg to produce an amount of fish two and a half times its own weight. But, on the other hand, there are only 59 mgm. of water present in the original yolk, and this will be sufficient for only 70 mgm. wet weight of fish. And the observed weight of the fish at the end of the yolk conversion phase of development is actually about 150 mgm. The explanation of all this is, of course, that some of the initial dry material is lost during develop-

ment by combustion, and a good deal of the eventual water is taken in from outside. Now between a quarter and a third of the initial dry material is so used, so that the eventual dry weight in the finished fish is about 25 mgm., which at 84 per cent. water needs 155 – 25, i.e. 130 mgm. water. But, as there were only 59 mgm. water present in the yolk at the beginning, 130 – 59, i.e. 71 mgm., must have been absorbed from the exterior. These relationships are shown in the chart, constructed by Gray and given in Fig. 236. It will be seen at a

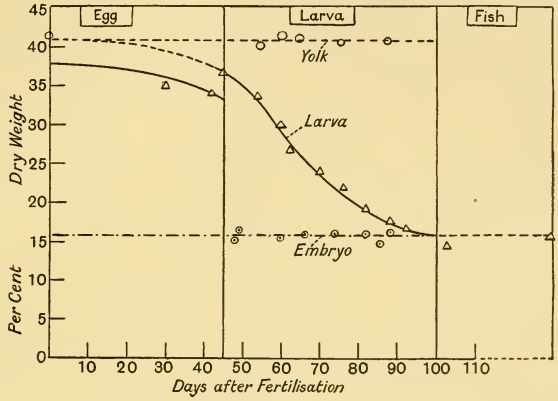



Fig. 235.

glance that the newly fertilised egg contains enough solid for constructing the finished embryo and for providing the material for combustion to serve the basal metabolic requirements, but it does not contain even half enough water. The latter has, therefore, to be taken from the aqueous environment, according to the following generalised equation:

| Wet yolk (1·0 gm.) | + | External water (0·7 gm.) | = | Wet fish (1·56 gm.) | + | Dry yolk used for other purposes (0·14 gm.) |
|---|---|---|---|---|---|---|

By respiration experiments, as already remarked, Gray was able to account for all the yolk disappearing in the last of these fractions.

In a later paper Gray found that a peak exists in the wet weight of the larva (yolk plus embryo); this is illustrated in Fig. 237. Up to the 85th day the wet weight of the larva increases, but after that time it falls, although the wet weight of the embryo steadily increases throughout the period. Thus after the 85th day the yolk is

being used by the embryo faster than it can absorb water from outside, and this begins when about 1·10 gm. of yolk is left unconsumed. This is important with regard to growth-rate (see p. 406).

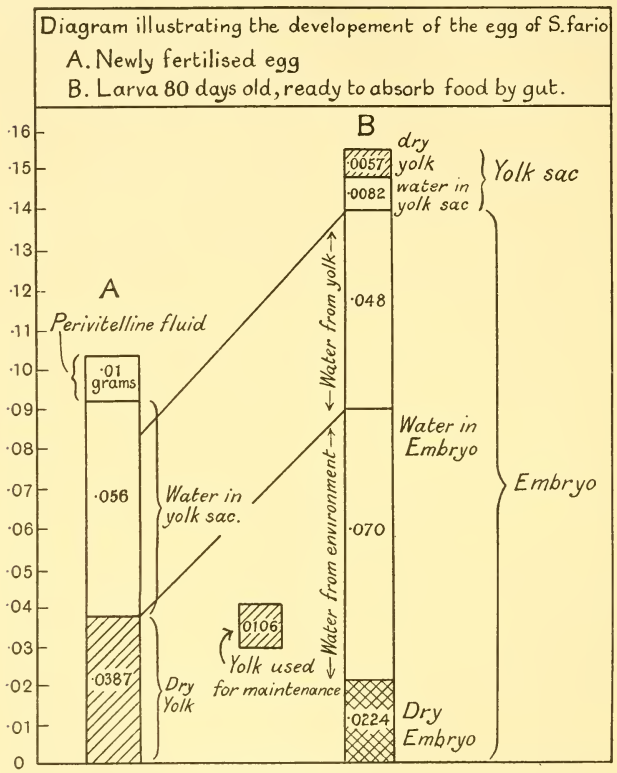



Fig. 236.

Ranzi's parallel work on the egg of the squid, *Sepia officinalis*, led to an equation similar to that given above for the trout:

| Wet yolk (1·0 gm.) | + | External water (0·78 gm.) | + | External ash (0·033 gm.) | = | Wet squid (1·727 gm.) | + | Dry yolk used for other purposes (0·089 gm.) |
|---|---|---|---|---|---|---|---|---|

He obtained curves identical in general form with that shown in

Figs. 232 and 235, the water-content of the whole system rising, that of the yolk remaining steady and that of the embryo slightly falling (82 to 75 per cent.). The total weight of this egg rises considerably, from 76·9 to 132·8 mgm. and as will be shown later, ash as well as water is absorbed during development. The undeveloped egg of

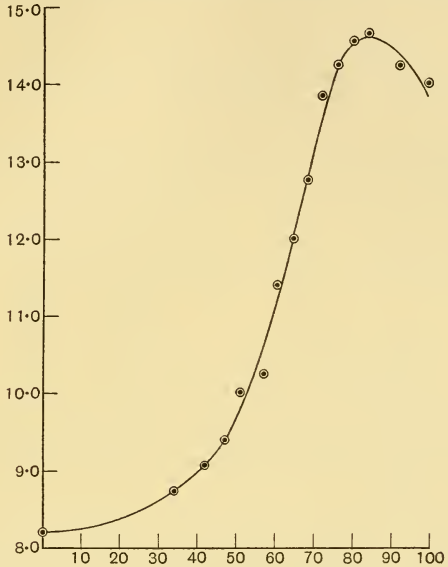



Fig. 237.

*Sepia* contains 40·4 mgm. of water but the hatched embryo 100·6 mgm. so that 60 per cent. of the final amount must have been taken in from the sea. The percentage of water in the system correspondingly rose from 52·5 to 75·8[1].

The work of Weismann indicated a similar absorption of water during the embryogeny of the cladoceran, *Daphnia*, and this was con-

[1] An equation for the egg of the axolotl, *Amblystoma*, can be derived from Dempster's data :

| Wet yolk | + | External water | = | Wet axolotl | + | Dry yolk used for other purposes |
|---|---|---|---|---|---|---|
| (1·0 gm.) | | (3·92 gm.) | | (4·84 gm.) | | (0·086 gm.) |

Ranzi's figures apply to the pre-hatching period only, Dempster's apply to the whole yolk-sac period as well.

clusively demonstrated by Ramult for *Daphnia*, *Ceriodaphnia*, *Scapholeberis*, and *Simocephalus*.

Another case in which it has been shown that the egg contains enough solid but not enough water for the finished embryo is that of the ovoviviparous batoid elasmobranch, *Torpedo marmorata*. Davy found as long ago as 1834 that the mean weight of the egg when undeveloped was 182 grains, that of the egg plus early embryo was 177 grains, while that of the finished embryo was 479 grains. Allowing 80 per cent. of water for the mature state, which is very reasonable, only 95 grains would be required of non-combusted solid, so that it is very probable the increase was all due to water, especially as the gelatine-like egg-cases would hardly allow anything but water to pass through them. Vidakovich afterwards found an increase in weight of the finished embryo over the egg, of 40 per cent., and from my own observations of the fish on which he worked, *Squalus acanthias*, I believe that the events which take place there are quite analogous to those in the trout, thus:

| | Water (gm.) | Solids (gm.) |
|---|---|---|
| Undeveloped egg ... ... | 14·2 | 8·8 |
| Finished embryo ... ... | 35·0 | 6·0 |

Parker & Liversidge, again, reported that the undeveloped eggs of *Mustelus antarticus*, an ovoviviparous selachian, measured 43 × 16 × 10 mm. (roughly) while the ripe embryos ready to hatch measured 220 × 25 × 25 mm.

A very remarkable case is that of the (Siluroid) catfishes which incubate their embryos in their mouths. Wyman in 1857 studied several species of the genus *Bagrus* at Paramaribo in Dutch Guiana, and found that the hatched embryos not yet liberated from the parental mouth weighed considerably more than the undeveloped eggs. His conclusion that a nutritive fluid was supplied does not of necessity follow ; it is likely that a good deal of water was absorbed.

Again, in gastropods the eggs swell greatly, according to Nekrassov. As regards crustacea Needham & Needham, in the course of other work on the chemical embryology of the sand-crab, *Emerita analoga*, observed that the water-content of the whole egg was 63 per cent. in the cleavage stages, 75 per cent. about the middle of development, and 85 per cent. at the time of hatching. But the extreme case is undoubtedly the desiccation that phyllopod (e.g. *Artemia salina*) and cladoceran eggs may go through before their development. Wolf found

that phyllopod eggs would withstand 14 years' drying in a desiccator without losing their power of development and Pirie has exposed *Artemia* eggs to phosphorus pentoxide at less than $\frac{1}{2}$ mm. pressure for several days after which development proceeded normally. Carpenter noted much the same facts in the case of rotifer eggs. When they are put into water they develop: *all* the water in the body of the embryo being derived from the environment except that small trace which is bound to the hydrophilic colloids of the egg (see Gortner & Newton, and Robinson). Doubtless this hardiness is an adaptation to the seasonal drying up of the pools in which these animals live.

There is strong reason to believe, therefore, that the eggs of aquatic animals do not contain enough water to make their end products, but have to absorb it from their surroundings. It is possible that some obscure phenomena may receive an explanation on these grounds; thus the eggs of the pike (*Esox*), according to Kasanski, rotate round and round within their membranes, after only 24 hours cleavage; and this movement, which continues until the muscles are formed, may be an adaptation for ensuring water-intake, by setting up currents within the egg-case. And Amemiya states that the eggs of the fresh-water teleost, *Oryzias latipes*, show conspicuous undulating movements of the blastoderm from an extremely early stage onwards. Giard, long ago, showed that many aquatic eggs (fresh-water molluscs, Hirudinea, marine polychaetes, molluscs and nudibranchs) would develop well if kept in moist trays, not actually immersed in water, but that moisture was essential. Certainly in many cases, as we have seen, the percentage dry weight of aquatic eggs is much higher than that of the corresponding fully formed tissues.

"If then", said Gray, "it may be assumed that most, if not all, aquatic organisms are dependent on the environment for a supply of water, an interesting problem of phylogeny is opened up. The primitive vertebrate is, with good reason, regarded as the offspring of an aquatic type, but at some stage in the history of the truly terrestrial animals there must have come a time when oviposition occurred on land and not in water. During the earlier stages of their evolution terrestrial vertebrates no doubt laid their eggs in water where possibly the factor of greatest survival value consisted in the newly hatched individual having reached a stage at which it might fend for itself and be of active habit. Now an animal such as the trout necessarily hatches at an early stage since the increasing volume

of the larva soon reaches the volume of the original egg-shell; further development without hatching would crush the embryo against the egg-membranes. In aquatic animals a postponement in the date of hatching is only possible when either the original egg-membrane is highly elastic or when it is separated from the yolk by a wide perivitelline space. Now an aquatic egg containing sufficient perivitelline space to allow the act of hatching to be postponed until the young organism is fully formed (and comparatively unhampered by remaining yolk) would form just the system most easily adaptable to terrestrial habits. Such an egg laid on land would undergo its normal development as long as it was protected from undue evaporation during the incubation period. Thus the experimental data we have point strongly to the suggestion that the Reptilia and their derivatives arose from a type of organism whose eggs were laid in water but which did not hatch until the yolk-sac period of development had reached an advanced stage. The wide perivitelline space which was then a necessary feature for the accommodation of the enlarging embryo became on land the means whereby water was supplied for development. The significance of the white of the hen's egg can be realised by the fact that after 19 days of incubation the embryo has absorbed 10 cc. of water from it. The eggs of all birds and of many reptiles are of the same type, but in some reptiles the necessary water is partially supplied from external sources, and the eggs swell appreciably after being laid" (e.g. *Sphenodon*—Dendy; *Dermochelys*—Deraniyagala; and many others).

A remarkable illustration of this is supplied by the work of Karashima on the Japanese marine turtle, *Thalassochelys corticata*, which lays eggs the size of pingpong balls in the damp sand above high-water mark. He did not himself calculate the movements of water in these eggs but this can easily be done from his figures, with the following result:

Table 105. *Water in grams per* 100 *eggs.*

| Days of development | White | Yolk | Embryo | Allantoic and amniotic liquids | Total | Absorption |
|---|---|---|---|---|---|---|
| 0 | 1330 | 1381 | — | — | 2711 | |
| | | | | | | 87 |
| 15 | 699 | 2096 | 3 | — | 2798 | |
| | | | | | | 78 |
| 30 | 520 | 1783 | 131 | 442 | 2876 | |
| | | | | | | 971 |
| 45 | 0 | 225 | 1262 | 2360 | 3847 | |
| Hatched | — | — | 1655 | — | — | — |

This table demonstrates (*a*) that the chelonian yolk absorbs water from the white, just as does that of the bird—a process which will tend to dilute the yolk appreciably if it goes on faster than the formation of embryo and amniotic liquid; and (*b*) that 1136 gm. of water are absorbed from the exterior by 100 eggs, i.e. 42·0 per cent. of the original amount provided by the maternal organism, which thus expects its embryos to obtain for themselves about a third of the water they require. A swelling of the egg must certainly have taken place, though Karashima did not report it: and this lack of water may in part account for the relatively small size of many chelonian eggs.

Still more remarkable is the fact, reported by Cunningham, that another turtle (from North Carolina), *Chrysemys cinerea*, has a special mechanism for wetting the earth in which the eggs are to incubate. "These turtles", says Cunningham, "select high ground in which to build their nests, sometimes a considerable distance from water. Usually the ground chosen is hard and dry, but a sandy beach may be used. The dirt is first moistened by water from a supernumerary bladder; as is shown by the fact that the dirt in the hole and surrounding it is wet while that further away is hard and dry." Cunningham analysed the water in this bladder (the function of which had previously been unknown) and found it to be a dilute urine, containing only 0·0005 per cent. of nitrogen. During development the eggs swell somewhat. This is a notable link in the evolutionary chain, for here the turtle goes out of its way to provide a store of water for its terrestrial eggs, yet *outside* not inside them. This must be the furthest point to which a non-cleidoic egg could go in a terrestrial environment (see p. 1103). Moulton states that terrapin eggs have been commercially incubated by putting them in sand and sprinkling the surface every week until hatching, and Hochstetter reports success by a similar method on *Emys europaea*. Deraniyagala and Hildebrand & Hatsel find that loggerhead turtle's eggs (*Caretta*) take up a good deal of water during incubation. "Apart from other advantages", Gray continued "the value of the invention of viviparity is obvious; it entirely removes the necessity for the parent organism to provide in the newly fertilised egg enough water to last the embryo throughout the whole developmental period. Now it is known that the vitelline membrane in the hen's egg is permeable to water but not to salts and other osmotically active substances. Were the yolk surrounded

by pure water, the latter would rapidly pass into the yolk and thereby produce a large and mechanically weak ovum. If the aqueous surroundings, on the other hand, consisted of crystalloid substances the ease with which the embryo could obtain water would become increasingly less with increasing age owing to the rising osmotic concentration of the external salts. Since, however, the initial osmotic equilibrium between embryo and yolk on the one hand, and the aqueous surroundings on the other, is effected by means of a colloid, then although water is withdrawn from the latter its osmotic pressure does not rise as much as would that of a solution of a crystalloid. The existence of an albuminous solution round the yolk of terrestrially developing eggs appears to be an admirably adapted mechanism for providing the growing embryo with water." Thus Gray's theory amounts to this, that we may see in the egg-white of the hen's egg a mechanism for supplying the embryo with a relatively constant pressure-head of water. If the chick embryo were dependent on the yolk alone, it would never be able to construct its tissues, for the yolk has only about 45 per cent. of water and the chick about 80 per cent. at the time of hatching. It is hardly necessary to indicate the way in which the findings of Vladimirov, referred to above (p. 880), fit in with the general viewpoint of Gray, and it looks very much as if the acid which seems to be produced by the embryo and which appears in the white, is the regulatory or control device governing the transfer of water from egg-white to embryo.

Table 106 gives a succinct survey of the movements of water in the hen's egg; it was calculated by Gray from the experimental data of Murray. It clearly appears that at least two-thirds of the water in the finished chick embryo is derived from the albumen.

In a later paper Gray continued his exposition of the relations between the water metabolism of the embryo and the evolution of terrestrial vertebrates. Agreeing with Watson that the main problem of evolutionary modifications centres round the possibility of deriving one morphological type from another without requiring any functional discontinuity of the organs involved, he considered the origin of the egg-white in birds. No reptilian or avian egg exists without an albuminous phase, and we may assume that its function is not radically different from that of the egg-white of the chick. The amniota, then, solved the problem of providing their embryos with

an adequate water-supply by coating their eggs with a watery protein-containing mass. "Now it is hardly conceivable", said Gray, "that the albuminous layer of the amniote egg arose *de novo* as an adaptation to terrestrial life, for this would involve a sudden change in the structure of the oviduct. By the principle of

Table 106.

Survey of the movements of water in the hen's egg.

| Day of development | Water in grams: Present in embryo | Present in yolk | Present in egg-white and amniotic liquid | Lost from whole egg by evaporation at 23 % humidity | Gained by whole egg by combustion of fatty acids in yolk |
|---|---|---|---|---|---|
| 0 | 0·0 | 8·5 | 29·9 | 0·0 | 0·0 |
| 6 | 0·4 | 8·45 | 27·2 | 2·4 | 0·01 |
| 8 | 1·1 | 8·4 | 25·4 | 3·5 | 0·05 |
| 10 | 2·5 | 8·2 | 23·0 | 4·6 | 0·12 |
| 12 | 4·6 | 7·8 | 20·4 | 5·6 | 0·27 |
| 14 | 7·9 | 6·9 | 16·9 | 6·7 | 0·50 |
| 16 | 12·0 | 5·3 | 12·6 | 7·8 | 0·80 |
| 18 | 18·1 | 2·3 | 9·2 | 8·8 | 1·20 |
| 20 | 27·4 | 1·0 | 2·2 | 9·8 | 2·00 |
| | | 7·5 | 27·7 | Lost from yolk and white respectively | |
| | | | 35·2 | Lost from both | |
| — | — | — | 35·2 | | |
| | | | 9·8 | | |
| | | | 25·4 | Lost other than by evaporation + 2·00 by synthesis = 27·4 of which, even if the whole of the water of the yolk went to form the embryonic tissues (which is unlikely) 20·0 gm. or 73 per cent. would be water passing from egg-white to embryo. | |

Compare this Table with Table 105; whereas the avian egg loses some 25·5 per cent. of its initial store the chelonian egg gains some 40·0 per cent. from the exterior. For the question of bound water, see p. 879.

physiological continuity it is much more reasonable to suppose this layer as equivalent to such homologous structures as are found in the anamniota. Among fishes tertiary egg-membranes rich in water are found in the Dipnoi, and they appear to be present in all amphibia. These membranes apparently protect the egg against destruction by predatory animals though they may have subsidiary functions associated with the incubation of the embryo. In most amphibia, where the tertiary envelope is of a mucoid nature, the full

water-content of the envelope is not attained until after the egg has been deposited in water, but interesting and suggestive modifications are found in the eggs of those amphibia which deposit their eggs on land. In *Phyllomedusa* and in *Rhacophorus* the protective function of the mucoid envelope is to a large extent replaced by other devices, and it is difficult to resist the conclusion that the envelopes themselves are largely devoted to the provision of water. In *Phyllomedusa hypochondrialis* the eggs are deposited in the folds of leaves. The mucilaginous egg-capsules rapidly liquefy after oviposition and provide a fluid medium in which the eggs develop. Agar observed that a certain percentage of capsules contain no eggs, and this suggests that the function of these membranes is to augment the amount of water available for the larva. The essential point is that the whole of the water necessary for development is provided by the walls of the maternal oviduct. Similarly the eggs of *Rhacophorus schlegelii* are laid in a subterranean burrow. Having formed this burrow, the female secretes into it a mucilage which with the aid of her feet is rapidly worked into a froth. Into this froth the eggs are laid and as development proceeds the froth is gradually liquefied. Here again all the water for development is derived from the female organs. From these types it is not difficult to derive either the egg of a reptile with its solid albumen phase or the egg of a bird with its fluid albumen which has entirely lost the power of protecting the embryo against predatory foes. It is interesting that far from requiring a supply of water from external sources, the eggs of birds fail to develop unless a certain amount of water is lost by evaporation during incubation, as Chattock has shown. And a suggestive experiment of Weldon's, who incubated eggs in such a way as to replace the amount of water normally lost by evaporation, indicates that the proper formation of the amnion is dependent on loss of water by evaporation.

"Since the mammals are derived from the oviparous reptiles it is of interest to consider how the small eutherian egg can be derived from that of the latter group without any break in the physiological functions of the organs concerned. A conceivable line of origin is suggested by the eggs of monotremes. These have no true albumen layer and the yolk ovum lies close under the shell. As it leaves the ovary, the egg is about 2 mm. in diameter, but during its passage down the oviduct its bulk is enormously increased so that the yolk is about 14 mm. in diameter before the shell is deposited (Caldwell).

This 300-fold increase in volume must largely be due to absorption of water, though a certain increase in dry weight may well occur. The only significant difference between an egg of a monotreme and an egg of a reptile is that, in the former, the aqueous secretions of the walls of the oviduct are passed straight into the yolky ovum itself instead of being deposited on its surface as a separate phase. In eutherian mammals this process has gone one step further since the water contained in the mother's blood is passed, not into the ovum, but direct into the embryo.

"If these arguments are sound", Gray continued, "there seems good evidence to show that terrestrial vertebrates have descended from a fish-like ancestor which possessed a glandular oviduct. The secretions of these oviducts were at first utilised as a protective covering to the eggs, but eventually they made it possible for the eggs to develop on land by providing an adequate supply of water to the embryo."

Gray's contention that there exists an evolutionary continuity between the amphibian egg-jelly and the avian egg-white, that they are, in fact, homologous structures, acquires still further interest from an isolated observation reported by Banta & Gortner. In the course of their work with *Amblystoma*, they found one day a mass of eggs contained in an opaque milky white jelly instead of the usual transparent and translucent material. Investigation showed that the dry weight of the normal jelly was 337 mgm. per cent. and that of the milky white one 361 mgm. per cent. When desiccated the jellies were indistinguishable in appearance but swelled up again to their original condition. The phenomenon was not, as far as could be ascertained, due to bacteria, and as the milky jelly has 9·18 per cent. nitrogen (dry weight) as against the 8·32 per cent. of the normal kind, they thought that perhaps the former might consist mainly of albumen instead of mucin. This was confirmed by qualitative tests. Here then was an aberrant example of an amphibian egg-jelly resembling the avian and reptilian egg-white, and indeed only requiring a shell to be transformed into it[1]. It affords an interesting commentary on Gray's remarks. The suggestion of Steudel & Osato mentioned on p. 331 may also be recalled—they pointed out that mucoprotein is not absent from the egg-white even of birds, and expressly referred to an evolutionary continuity with amphibia. The land-frogs offer some

[1] Shells of a rudimentary kind do occur in amphibia (e.g. the land-frog *Rana opisthodon* and the African toad *Xenopus laevis* (Bles)).

attractive material for the study of these questions, e.g. the *Eleutherodactylus* of Dominica described by Howes, which lays large transparent crystal-clear eggs and has no external tadpole stage.

It is not without interest that these watery mucilaginous envelopes are found elsewhere in the animal world, e.g. in the eggs of certain insects, mostly *Trichoptera* (caddis-flies) and midges of the *Chironomus* class[1]. The nature of the protective function served by these jellies raises problems of much interest. I have already suggested that the reason why bacteria attack the amphibian egg-jelly so slowly is that it is practically a pure protein, mucin, and that a certain proportion of protein breakdown products is essential for good bacterial growth. There is also much evidence that similar properties may be ascribed to the avian egg-white. There is a large literature on the dietetic aspect of raw and cooked white of egg which may be found summarised in the work of Bateman but without going into it at this point, it may be remarked that raw egg-white is very resistant to digestive enzymes, containing a definite antitrypsin and an antipepsin. In Section 19·3, moreover, we shall see that a natural bacteriolysin is present in raw egg-white (see Sharp & Whitaker).

Insect eggs, indeed, provide further light on the evolution of terrestrial embryos; thus, work on the silkworm (*Bombyx mori*) brought out the following figures:

| | Farkas Water-content | Tichomirov Water-content |
|---|---|---|
| Unincubated eggs ... ... ... | 64·56 | 64·49 |
| Finished larvae ... ... ... | 71·78 | 69·80 |
| Unused material, membranes, etc. ... | 13·98 | — |

In the case of this insect, at any rate, the finished larva has a little more water in it than the original egg, but the explanation may lie in the fact that at hatching a notable quantity of very dry material is left behind. During metamorphosis also, the organism seems to become richer in water, but here again a mechanism involving the production of dry membranes, cocoon, etc., is perhaps responsible.

It is possible, however, that the eggs of some insects, like those of reptiles, though apparently self-contained, take in water. Thus Wheeler informs us that ants "salivate" over the eggs in their communities and suggests that this saliva may be absorbed. Similarly, Weyrauch reports that the earwig (*Forficularia*) licks its eggs after laying them; and if this is not done they will not develop.

[1] Also in fishes (e.g. *Lepidosiren* (Carter & Beadle), where it disappears early in development and is perhaps functionless).

And a good deal of evidence exists, indicating that insect eggs, although terrestrial, need a humid environment for proper development. Thus Harukawa obtained the following figures on the Oriental peach-moth:

| Relative humidity | Percentage of eggs hatching |
|---|---|
| 10 | 49·4 |
| 35 | 83·1 |
| 65 | 100·0 |

and it is known that the Trinidad froghopper, *Tomaspis saccharina*, cannot hatch at all under 90 per cent. humidity. Again, Peterson has shown that *Aphid* eggs are very dependent on their normal humidity for proper hatching, which is easily stopped by a small decrease in the water-content of the environment (*Aphis avenae* and *Aphis pomi*) and Dampf, Hoffman & Varela have shown the same thing for various grasshopper's eggs. (See also Tchang and Andersen.)

It remained for Bodine to show in 1929 that the water-content of the whole system in grasshopper and other orthopteran eggs rose during development. This process was evidently closely associated with the egg's metabolism for increase of temperature accelerated the water-intake. We may therefore have to picture the insects as solving the problem of terrestrial embryonic life, not by providing enough water in the eggs from the maternal body, as the sauropsida do, but by inventing a sort of *deliquescent* egg which should absorb atmospheric moisture. In this connection, Peacock has made some suggestive experiments on the eggs of the saw-fly *Pristiphora pallipes* which normally inserts them in pockets artificially contrived in gooseberry-leaves. It seems very probable that water is absorbed by the eggs from the plant, for Peacock found that if the stalk of the gooseberry-twig was immersed in a weak solution of eosin, the dye would pass into the leaves and thence into the eggs. On the other hand, if the eggs were removed from the pockets at the beginning of development they hatched as usual, being able, apparently, to pick up from the air all the moisture they required, and swelling normally. Swelling, in fact, seems to be a regular occurrence in the development of the eggs of all Tenthredinidae, Cynipidae, and Formicidae. Kerenski has shown that the eggs of the scarab beetle, *Anisoplia austriaca*, double their wet weight in a fortnight and that this increase can be at the expense of distilled water, no dissolved substances being taken up, and development proceeding normally. As insect eggs are so small, they no doubt make use of "micro-climates", for small crevices etc. may have a very different humidity from the main climate in which they exist.

## 6·7. Water-metabolism in Aquatic Eggs

Fig. 238 taken from Kronfeld & Scheminzki's paper illustrates once again the relations we have been discussing in the case of the trout egg. It shows that the maximum intensity of water absorption occurs about half-way through the yolk-sac free-swimming period. The total water in the larval system rises steadily from fertilisation onwards and also that in the embryo, but there is a distinct loss of water from the yolk. This does not mean that the yolk becomes drier, but is simply a measure of the disappearance of the yolk. Before hatching the loss of water from the yolk is just compensated for by the gain in water of the embryo, showing that at first the system is a closed one. There is here a certain contrast with the frog's egg, for the water content of the embryo plus yolk there begins to rise well before hatching, though Gray asserts that water-absorption begins before hatching in the trout too. In the lump-sucker (*Cyclopterus*) Hayes found no intake of water until after hatching, but in the Atlantic salmon (*Salmo salar*) there was a definite rise of about 10 per cent. Kronfeld & Scheminzki fully appreciated the fact that the trout egg contains enough solid but not enough water to make the embryo, and they attempted to show that at the end of the first period a slowing of the growth-rate was perceptible, owing to the inability of the embryo to get sufficient water through the egg-membranes to dilute its solid material. The figures on which they based their growth-rate estimations, however, were rather too few to substantiate this; nevertheless, some statements in Gray's paper appear to coincide with it, and

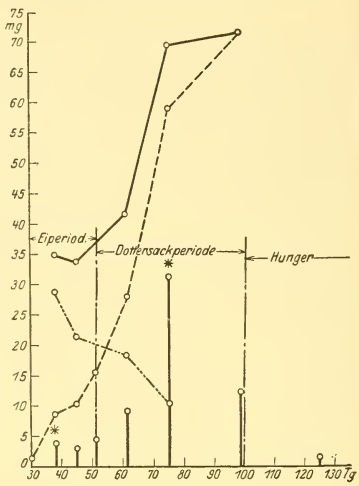



Fig. 238. The vertical lines indicate relative intensities of water absorption, and the asterisks the maxima of dry and wet weight respectively.

provisionally it may be accepted. Fig. 239 gives a graph plotted from their data. The percentage growth-rate falls markedly at the end of the first period, only to rise again to a maximum during the free-swimming period. Everything, in fact, points to a resumption of the growth-process as soon as an unlimited supply of water is available, which will only fall off again when the nutriment in the yolk-sac is beginning to be exhausted. Kronfeld & Scheminzki pointed out that the decline in the growth-rate before hatching occurred before all the water in the yolk was finished, as would be expected in view of the osmotic pressure of the latter. They drew attention to Schaper's well-known experiment on frog embryos and larvae, in which he placed them in salt solutions, and, by thus holding back the water which they needed to absorb to form their tissues, succeeded in inhibiting their growth. Kronfeld & Scheminzki confirmed this for the trout in some preliminary experiments. The question of whether growth of the trout fry can continue after the yolk has been used up was left open by Kronfeld & Scheminzki, but Weiss had previously maintained that this could occur, and Podhradski & Kostomarov confirmed Weiss' results on carp alevins at the end of their yolk-sac period. It must be supposed that this growth is either due to the persistence of small amounts of yolk which gradually get used up after the yolk-sac has apparently disappeared, or to the utilisation of muscles or other tissue as nourishment instead of yolk. The latter process is suggested by the work of Krzinecki & Petrov[1].

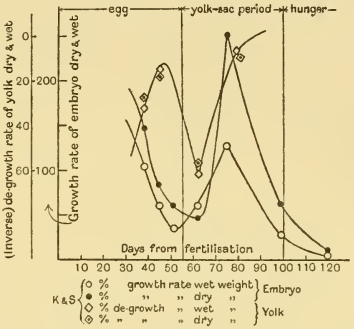



Fig. 239.

The process of water-absorption by the amphibian larva during its development was studied also by Bialascewicz, who used the unsatisfactory but relatively easy method of measuring volume changes. In all his curves, a temporary reduction of volume is seen about

[1] In certain cases, the intake of water by the egg before hatching will mask the loss of dry solids by combustion, if the eggs are weighed in air. Weighing in water, as suggested by Ritter & Bailey, may therefore be a useful method.

the second hour; this is due to the formation of the perivitelline fluid (see p. 784). According to Bialascewicz, this temporary reduction and the formation of the perivitelline space only occurred in fertilised eggs, as Fig. 240 shows, but perhaps the difference is quantitative rather than qualitative. In the later stages Bialascewicz measured the volume directly instead of calculating it from the measured diameter, and his results appear in Fig. 241, taken from his paper, where the average volume of one larva is plotted against the time in days. The shape of this curve is of interest, inasmuch as there is first of all a marked rise, followed by a comparatively stationary period during the last few days of the pre-hatching period, and then by a tremendous rise as soon as the larva has come forth from its jelly, and nothing interposes itself between the tissues and the water but the skin. These data are obviously in close agreement with the findings of Kronfeld & Scheminzki and of Gray. Thus Bialascewicz found that between the 2-cell stage and the blastula stage there was an increase in volume of (average) 10·6 per cent., and between the blastula stage and the gastrula stage an increase of 7·6 per cent. Fig. 242 shows the relative speed of volume increase at the different stages expressed in terms of volume increase of 1000 larvae in hourly periods. The higher level at gastrulation

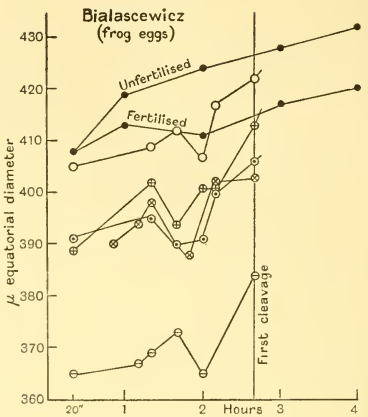



Fig. 240.

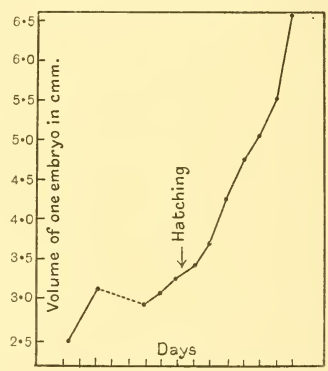



Fig. 241.

was related by Bialascewicz to the presence of the blastopore. The slight diminution in absolute volume between the 50th and 118th hours is reflected on the speed curve by a big drop, but after that the curve rises with only minor variations. Both Davenport and Schaper noted an increased growth-rate in the frog larva after hatching, so that the results of all the workers on the frog embryo are in agreement with those of all the workers on the trout. The whole mass of data shows clearly an absorption of water diluting the yolk as it is transformed into the tissues of the embryo.

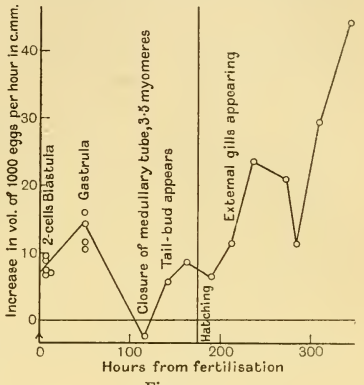


Fig. 242.

Bialascewicz also investigated the part played by the jelly of the frog's egg after hatching as a source of solid and water for the embryos. Frog larvae are nearly always to be seen hanging on to the jelly with their suckers after having hatched, and Bialascewicz rightly thought it not improbable that the jelly might contribute something to the larvae. Measurements certainly supported this; thus the average weights of larvae he found to be as follows:

| | Weight in milligrams | |
|---|---|---|
| | Wet | Dry |
| 8 days ... ... ... ... ... | 9·52 | 1·15 |
| 26 days (in distilled water) ... ... ... | 25·24 | 1·08 |
| 26 days (in distilled water but plus the jellies) | 84·46 | 4·22 |

This was a striking demonstration of the absorption of water. In the second instance much water had been absorbed, but the dry substance was the same in amount, or rather slightly reduced owing to combustion of yolk, whereas if the jellies were present the dry substance was much increased as well as the water. The loss of dry substance from the larvae was also found by Bialascewicz for the early stages; thus, between the 1st and the 4th day, about 5 per cent. of the dry weight was lost. He measured the effect of temperature on the increase in volume of frog's eggs at different stages, and came to the conclusion that temperature had no effect on the amount of water

absorbed by the larvae, though the time they took to absorb a definite amount was, of course, made longer or shorter. The effect of rising temperature on the unfertilised eggs was to increase the permeability of their membranes to water, so that, for instance, at 10°, an egg in a definite period would increase its volume by 0·05 c.mm., and at 20° by 0·28 c.mm. But the possibility that the increase with temperature was really due to the production of an unusually large amount of osmotically active substances in the egg-protoplasm was not excluded. Bialascewicz argued that as the permeability to water is increased five times by a rise of temperature of 10°, and as the development rate is increased only two and a half times, one would expect to find more water in larvae brought up at 20° than at 10°. Since experimentally this was not the case, Bialascewicz concluded that the permeability of the membranes to water was not the determining factor in the absorption of water during amphibian embryonic growth.

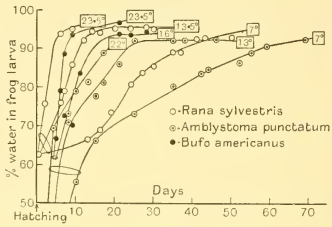


Fig. 243.

Galloway, inspired by Davenport's early work, also made a study of the effect of temperature upon the absorption of water by the developing frog larva. The embryos of *Rana sylvestris*, *Amblystoma punctatum* and *Bufo americana* were subjected to temperatures varying from 6° to 25°, and the water-content estimated from time to time. The results obtained are shown plotted in Fig. 243, whence it can readily be seen that the warmer the environment, the more rapidly did the imbibition of water go on. But Galloway found that at higher temperatures the final amount of water in the body was slightly more than that at lower temperatures although the developmental process up to the point when 75 per cent. water was reached was not so much retarded by lower temperature as the stage representing the maximum percentage of water. (See Table 107.) The effects of temperature, therefore, were more marked on the second phase of the process than on the first. In the first stage, assimilation of yolk and cell-division is prominent, in the second stage, assimilation of water. This is in exact accord with the finding of Bialascewicz, for a period in which imbibition of water is

prominent would thus be expected to be more sensitive to temperature change than one in which it is not prominent. As it is difficult to see why an apparently physical process should be so much affected by temperature, we must suppose that the absorption of water is regulated by chemical means. Galloway observed a regulatory process at work, in that individuals which were placed at 13° for a week, and then changed to a warmer environment, showed a greater increase of water than those which had been in the warmer environment from the beginning.

Table 107.

| | Time in days required to attain 75 % water | | | Time in days required to attain 95 % water | | |
|---|---|---|---|---|---|---|
| | Highest temp. | Medium temp. | Lowest temp. | Highest temp. | Medium temp. | Lowest temp. |
| *Rana* ... ... | 2 | 5·7 | 20 | 5 | 28 | 50 |
| *Amblystoma* ... | 9 | 12 | 27 | 21 | 50 | 70 |
| *Bufo* ... ... | 5·5 | 7·5 | — | 14 | 32 | — |
| Averages ... | 5·5 | 8·4 | 23·5 | 13·3 | 36·6 | 60 |
| Retardation in days, reckoned from time required at highest temperature | — | 2·9 | 18 | — | 23·3 | 46·6 |
| Retardation similarly calculated in % | — | 53 | 327 | — | 175 | 350 |

The work of McClure provides an interesting commentary on the great absorption of water by the developing amphibian egg. He found that in the early cleavage and medullary groove stages of *Bufo* and *Rana* no cell will store trypan blue. But about the time of hatching (9 days from fertilisation) a change takes place and storage of the dye goes on vigorously, as it is taken up from the solution surrounding the eggs. This is probably because the lymph vessels are formed about the 5th or 6th day from fertilisation and can take the dye granules with the incoming water from the periphery to the interior of the organism.

## 6·8. The Chemical Constitution of the Embryonic Body in Birds and Mammals

We may now return to the chick embryo. We have to consider under the heading of general metabolism a variety of data, among which the percentage constitution of the embryo at different stages of its development is among the most important. This leads

directly to the question of the absorption curves for the various substances, i.e. the determination of the relative intensities with which various substances are absorbed from the yolk or the non-embryonic parts of the egg. These problems, again, cannot be dealt with without discussing the efficiency with which the raw materials are transformed into the finished embryo at different stages. All these subjects can best be treated in relation to the egg of the hen, for it is there that they have been most carefully worked out; in fact, it is only in the case of the chick, where the embryo can be separated from the yolk from a comparatively early stage, that these problems have been even partially solved.

First, as regards the percentage constitution of the embryo. Table 108 summarises the accurate data which we have on this question. It may be doubted whether in a computation such as this, in which it is desired to compare active strengths of constituents in the embryo, it is advisable to include substances packed away in an immobile form. Glycogen would not come under this category, nor would the adipose tissue of fat depôts, but the keratin in the feathers does seem somewhat remote from the balance of reactions in the body. Murray, whose suggestion this was, estimated the amount of feather substance present each day after the 12th, and his figures are represented in Col. 7. He only gave these values, however, as percentages of the body weight, so the actual weights are seen in Col. 8, the corresponding amounts of keratin (assuming the feathers to be 90 per cent. keratin) in Col. 9, and the true protein, excluding the feather protein, in Col. 10. Col. 11 reproduces Col. 6 corrected for the feathers, and finally Col. 12 shows the fat and Col. 3 the carbohydrate in grams per cent. of dry weight.

The graphic representation of this table is to be found in Fig. 244. The curves make up an interesting assemblage, for all three have peaks, and it is important to observe where they come. The carbohydrate one occurs on or before the 5th day, the protein one on the 11th day, and the fat one on the 20th day. This is reminiscent of the order in which, as will be seen (p. 993), the intensities of combustion run; carbohydrate about the 5th day, protein at 8·5 days, and fat towards the very end of development. The way in which fat overtakes protein at the end of development was first noticed by Murray, and Riddle observed in the pigeons' egg a preferential absorption of fat from the yolk at that time.

Table 108. *Constitution.*

| Day | Total carbohydrate mgm. % Wet weight | Total carbohydrate mgm. % Dry weight | True protein nitrogen per embryo (mgm.) | True protein per embryo Col. 14 ×6·25 (mgm.) | True protein (mgm. % dry weight) | Feathers % of total weight (Murray) | Actual weight of feathers (mgm.) | Keratin contained in the feathers (mgm.) | True protein excluding feather protein | Col. 6 corrected for feathers | Fat (gm. % dry weight) | Ash (gm. % dry weight) | Cols. 3+6 +12+13 (gm. % dry weight) | Residual substances, 100 −Col. 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 0 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 3 | — | — | 0·154 | 0·96 | 66·3 | — | — | — | — | — | — | — | — | — |
| 4 | 200 | 3532 | 0·55 | 3·47 | 73·5 | — | — | — | — | — | — | — | — | — |
| 5 | 150 | 2831 | 1·1 | 6·87 | 58·5 | — | — | — | — | 58·5 | 14·5 | 14·7 | 90·5 | 9·5 |
| 6 | 127 | 2261 | 2·01 | 12·57 | 53·3 | — | — | — | — | 53·3 | 14·8 | 14·3 | 84·6 | 15·4 |
| 7 | 114 | 1940 | 3·77 | 23·65 | 55·0 | — | — | — | — | 55·0 | 15·2 | 13·8 | 85·9 | 14·1 |
| 8 | 110 | 1775 | 6·84 | 42·76 | 57·9 | — | — | — | — | 57·9 | 15·6 | 13·3 | 88·7 | 11·3 |
| 9 | 114·5 | 1761 | 11·3 | 70·64 | 59·8 | — | — | — | — | 59·8 | 16·1 | 12·8 | 90·5 | 9·5 |
| 10 | 119 | 1702 | 17·76 | 111·0 | 59·6 | — | — | — | — | 59·6 | 16·5 | 12·2 | 90·0 | 10·0 |
| 11 | 125 | 1624 | 27·88 | 174·3 | 60·3 | — | — | — | — | 60·3 | 17·0 | 11·6 | 90·5 | 9·5 |
| 12 | 148 | 1684 | 43·51 | 272·0 | 60·5 | — | — | — | — | 60·5 | 17·5 | 10·8 | 90·5 | 9·5 |
| 13 | 164 | 1627 | 67·22 | 420·2 | 60·9 | 0·30 | 20·5 | 18·4 | 401·8 | 58·3 | 18·2 | 9·8 | 90·5 | 9·5 |
| 14 | 199 | 1623 | 109·34 | 683·0 | 62·1 | 1·10 | 99 | 90·0 | 593 | 54·0 | 18·9 | 8·7 | 91·3 | 8·7 |
| 15 | 225 | 1539 | 169·83 | 1061 | 63·4 | 1·77 | 204 | 183 | 878 | 52·4 | 19·9 | 7·9 | 92·7 | 7·3 |
| 16 | 256 | 1559 | 240·67 | 1505 | 63·8 | 2·74 | 395 | 356 | 1149 | 48·7 | 21·1 | 7·5 | 93·9 | 6·1 |
| 17 | 290 | 1686 | 311·15 | 1941 | 62·6 | 2·81 | 506 | 456 | 1485 | 48·1 | 22·7 | 7·5 | 94·5 | 5·5 |
| 18 | 311 | 1762 | 375·61 | 2348 | 60·4 | 2·23 | 507 | 457 | 1888 | 48·6 | 25·2 | 7·7 | 95·1 | 4·9 |
| 19 | 328 | 1852 | 430·47 | 2690 | 57·0 | 2·02 | 540 | 480 | 2506 | 53·1 | 28·3 | 8·0 | 95·1 | 4·9 |
| 20 | 325 | 1915 | 477·8 | 2986 | 53·3 | — | — | — | — | — | 31·5 | 8·4 | 95·1 | 4·9 |

The carbohydrate, protein, fat succession seen in these constitution curves explains Murray's finding that the calorific value of the embryonic tissue increases with age (see p. 947). Before leaving Table 108, some interesting facts which emerge from the values of the last three columns may be briefly mentioned. Col. 13 gives the inorganic material in the embryo in grams per cent. taken from Murray, and together with Cols. 3, 6 and 12, which represent the carbohydrate, protein, and fat, it ought to add up to nearly the whole of the dry weight as experimentally measured. Actually, as Cols. 14 and 15 show, it only adds up to about 90 per cent., leaving 10 per cent. on an average for all the other substances associated with life, lipoids, sterols, cycloses, pigments, waxes, etc. It is interesting to see that this residual value declines steadily during development, as if in the earlier stages there were a higher percentage of substances not included under the heads: carbohydrate, protein, fat, ash. Of course, estimation errors will be included in the residual value, and these will usually lead to slight losses in the substance estimated. When accurate figures become available for the amount of lecithin and cholesterol in the embryo, for instance, it will be interesting to begin the compilation of a balance-sheet of the residuum.

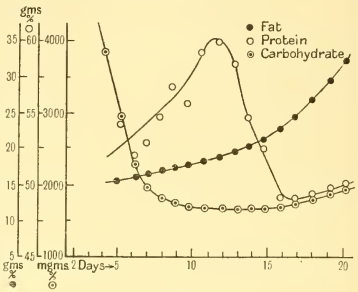



Fig. 244.

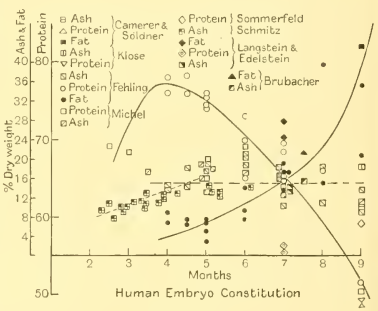



Fig. 245.

Whether the curves for percentage constitution in the chick embryo will be found to resemble those for other embryos we cannot as yet tell. For the mammalian embryo, however, a certain number of figures are available, though they in no way approach in thoroughness

the data which have been accumulated for the chick. It is possible to construct, from the analyses of Fehling and others on man, Michel on the rabbit, Liesenfeld on the dog and Hayes on the salmon, a table showing the percentage composition of the embryo, and, when

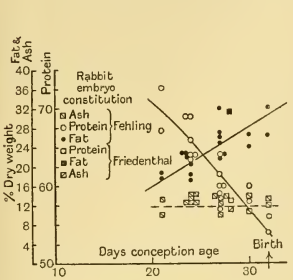



Fig. 246.

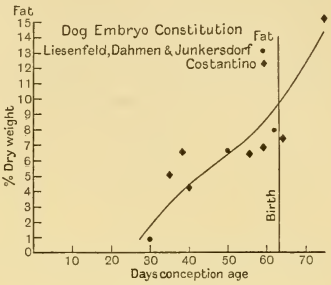



Fig. 247.

the data are plotted on a graph, Figs. 245, 246, 247 and 248 are obtained. Total carbohydrate cannot be considered, for the chick is the only embryo in which this has so far been estimated, and, in any case, it does not form a large part of the total solid there. But it is clear that both in the case of the human and the rabbit embryo there is just that cross-over between protein and fat which we find so markedly in the chick embryo. It is interesting that this was not noticed by the workers themselves, because they did not express their results in percentage dry weight, and all these inter-relations are, of course, obscured, if percentage wet weight is alone taken into consideration, owing to the sharply decreasing water-content. Next it is to be observed that Table 108 demonstrates a distinct lowering of the ash-content in grams per cent. during the development of the chick—this was the discovery of Murray—but the figures plotted in Figs. 245 and 246 do

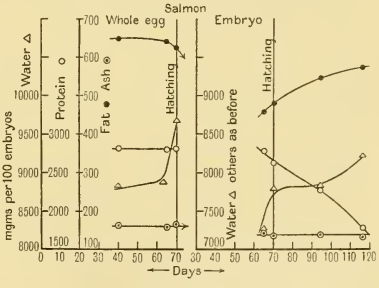



Fig. 248.

not show such a fall in the case of the mammalian embryo. Possibly this is due to the fact that we have no analyses of mammalian embryos in the earlier stages, and the earlier, sharper part of the inorganic curve may thus have been missed. Inspection of Col. 13 of Table 108 shows that this decline in ash-content percentage dry weight is in fact more rapid at the beginning than at the end of development. Some fragmentary data for the Jersey cow embryo contained in the paper of Moulton, Trowbridge & Haigh, do not seem to show any change between the 185th day of gestation and birth:

| Days of gestation | Percentage | | |
|---|---|---|---|
| | Fat | Protein | Ash |
| 185 | 14·6 | 74·5 | 11·0 |
| 232 | 14·0 | 69·0 | 17·4 |
| 279 | 13·7 | 69·0 | 17·4 |
| Birth | 14·8 | 69·0 | 16·0 |

Popov has also made analyses of cow embryos, but I have not been able to gain access to his data.

Another way of looking at the varying balance of the chemical constituents of the developing embryo is to enquire whether the ratios between any two of them change with age. This method was first used by Murray. "Before undertaking my experiments", he said, "I was impressed by what seemed to be a natural scale or gradient, as judged by various criteria, of the chief groups of substances under consideration, namely, salt, carbohydrate, protein, and fat. A tentative prediction was considered, that the following ratios would be found to decrease with age during ontogeny water/solid, inorganic/organic, carbohydrate/protein and protein/fat." The analyses which Murray and Needham subsequently made confirmed this prediction in every particular. Murray himself attempted to get the carbohydrate/protein ratio by estimating the amount of glycogen in the chick embryo at different stages, but

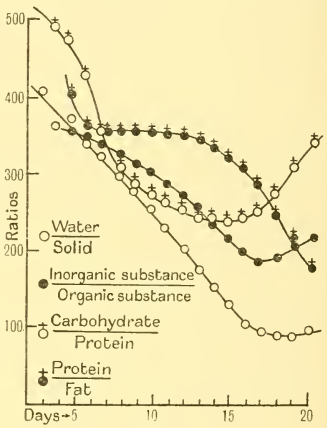



Fig. 249.

this was an unfortunate substance to choose as a representative of the carbohydrates, in view of the phenomenon of the transitory liver[1]. When I calculated this ratio on the basis of analyses of total carbohydrate, a definite decrease appeared, just as was expected. The bundle of curves which the plots of the ratios against the age give is shown in Fig. 249, and it is seen that they all pass downwards together.

Some of the minor points in this graph are worth considering. The decline in the ash/organic substance ratio, for instance, reminds one of the similar decline in the case of the frog embryo (see Fig. 230), and of Spek's suggestion that the velocity of cleavage of cells may depend, at any rate partially, on the concentration of electrolytes in which they find themselves. Perhaps we catch a glimpse here of a mechanism which controls cleavage velocity, for if by some means it were arranged that the ash-content of an embryo should fall with age, then the other factor might fall likewise, and the growth-rate would follow suit.

## 6·9. Absorption-mechanisms and Absorption-intensity

So far we have been considering the constitution of the embryo and how it changes with age, but the next question concerns the relative intensity of absorption at different times during its development. Up to the present time it has only been possible to make a start in this direction with the avian embryo, although morphologically a good deal is known about the various methods of absorption of the materials stored in the egg, and before considering the absorption intensity of the chick, something may be said about the absorption processes of other embryos. The principal treatment of this subject is that of Peter. It is evident that a fundamental distinction will here arise between holoblastic and meroblastic eggs, for if cleavage is complete and the whole egg divides into equal or nearly equal parts from the very beginning, the yolk will also get divided more or less equally, and being there already will not require any special means of transport into the constituent parts of the embryo. On the other hand, where cleavage is partial, and the vegetal pole of the egg does not divide at all, or where, as in the case of birds, 90 per cent. of the egg is vegetal pole, then mechanisms of various degrees of com-

[1] See Section 8·5.

plication have to come into play to supply the cells of the embryo with their yolk.

It is probable that the amount of yolk furnished to the egg by the parent has a good deal to do with determining whether its cleavage shall be complete or partial. Thus Peter's table (Table 109)

Table 109.

| | Diameter of egg in mm. |
|---|---|
| **Equal cleavage** | |
| Mouse (*Mus*) | 0·06 |
| Lanceolet (*Amphioxus*) | 0·1 – 0·13 |
| Man (*Homo*) | 0·15– 0·20 |
| Rabbit (*Lepus*) | 0·18– 0·20 |
| **Unequal cleavage** | |
| Toad (*Bufo*) | 0·6 – 0·15 |
| Lamprey (*Petromyzon*) | 1·1 – 1·2 |
| Newt (*Triton*) | 1·6 – 0·2 |
| Frog (*Rana*) | 2·0 |
| American bowfin (*Amia*) | 2·5 – 3·0 |
| Sturgeon (*Acipenser*) | 2·8 |
| Australian lungfish (*Ceratodus*) | 2·7 – 3·0 |
| Obstetric toad (*Alytes*) | 3·0 – 5·0 |
| African lungfish (*Protopterus*) | 3·5 – 4·0 |
| Salamander (*Salamandra*) | 3·5 – 5·0 |
| South-American lungfish (*Lepidosiren*) | 6·5 – 7·0 |
| Caecilian (*Hypogeophis*) | 7·0 – 8·0 |
| Caecilian (*Ichthyophis*) | 7·0 – 8·0 |
| **Partial cleavage** | |
| Sea-perch (*Serranus*) | 0·8 |
| Herring (*Clupea*) | 0·9 – 1·0 |
| Perch (*Perca*) | 1·4 |
| Duck-billed platypus (*Ornithorhyncus*) | 2·6 |
| Anteater (*Echidna*) | 3·0 – 4·0 |
| Garfish (*Lepidosteus*) | 3·0 – 5·0 |
| Trout (*Trutta*) | 4·0 – 5·0 |
| Trout (*Salmo*) | 6·0 |
| Lizard (*Lacerta*) | 9·0 |
| Snake (*Trochilus*) | 13·3 |
| Dogfish (*Pristiurus*) | 15–17 |
| Hagfish (*Bdellostoma*) | 20–30 |
| Torpedo (*Torpedo*) | 20–25 |
| Adder (*Pelias*) | 21–25 |
| Hen (*Gallus*) | 35 |
| Alligator (*Alligator*) | 40 |
| Ostrich (*Struthio*) | 105 |
| Porbeagle shark (*Lamna*) | 220 |

shows that the larger the egg the more likely it is to cleave only partially and to require special absorption methods, and the same thing is shown (roughly) by the relation between the size of the egg and the ratio between macromere and micromere sizes. In other words, the more yolk the egg has, the larger the yolk-laden macromeres will be compared to the micromeres.

| | Size of egg (diam. in mm.) | Ratio of micromeres to macromeres: $1/x$ $x$ |
|---|---|---|
| *Amphioxus* ... ... ... | 0·1–0·3 | 1·25 |
| *Petromyzon* ... ... ... | 1·1–1·2 | 1·88 |
| *Triton* ... ... ... ... | 1·6–2·0 | 1·33 |
| *Rana* ... ... ... ... | 2·1 | 1·4 |
| *Amia* ... ... ... ... | 2·5–3·0 | 7·7 |
| *Acipenser* ... ... ... | 2·8 | 8·0 |
| *Ceratodus* ... ... ... | 3·0 | 2·5 |
| *Salamandra* ... ... ... | 3·5–5·0 | 4·5 |
| *Lepidosiren* ... ... ... | 6·5–7·0 | 3·0 |

The nature of the yolk is also believed to influence to a large extent the form of cleavage. Thus yolk with large formed elements probably necessitates unequal divisions, and Hertwig was able by centrifuging, to make the frog's egg, which normally divides totally but unequally, divide partially.

Table 110.

| | Absorption | | | | | |
|---|---|---|---|---|---|---|
| | Direct through the division of the blastomeres | Merocytes | Yolk-cells | By the yolk-sac epithelium | By the intestine and the pancreatic juice | |
| Urodele amphibia (e.g. *Salamandra*) | + | − | − | − | − | Unequal cleavage |
| Gymnophiona (e.g. *Ichthyophis*) | + | − | − | − | − | |
| Protospondyli (e.g. *Amia*) | + | + | − | − | − | |
| Aetheospondyli (e.g. *Lepidosteus*) | + | + | − | − | − | Partial cleavage |
| Cyclostomes (e.g. *Bdellostoma*) | + | + | − | − | − | |
| Selachians (e.g. *Scyllium*) | + | + | − | − | + | |
| Telosteans (e.g. *Salmo*) | − | + | − | − | − | |
| Saurians (e.g. *Lacerta*) | + | + | + | + | − | |
| Aves (e.g. *Gallus**) | + | + | − | + | − | |
| Monotremes (e.g. *Echidna*) | + | − | − | + | − | |

* A complete account of the histology of yolk-absorption in the chick will be found in the monograph of Remotti. The yolk-absorption in cephalopods is very peculiar (see Portmann & Bidder).

The special absorption methods necessitated by partial cleavage are shown in Table 110. In the simplest type, shown by *Salamandra*, the macromeres gradually hand over their contents to the rest of the embryo. The appearance of merocytes and yolk-cells, situated

in, and absorbing, the yolk, complicates the process further. Then, as the mass of yolk becomes gradually more and more enormous relative to the embryo, the walls of a special yolk-sac take on absorptive functions, and in birds, for example, become of great importance. The monotremes show the abandonment of the intermediate methods[1].

We may now return to the rate of absorption of the yolk in the hen's egg.

An absorption-coefficient could not be calculated from the constitution figures only, for the rate of combustion of certain substances and the rate of transformation of others might, and in fact actually does, vary considerably. The only way to find out the relative absorption intensity of the various substances of the food-supply by the embryo is to make a large number of analyses and special calculations. So far the chick is the only embryo for which this has been possible. Murray's work provided the greater part of a sound foundation for these computations, and I made them in 1926 and 1927.

We may take first the absorption intensity of protein throughout development. To calculate it, it was necessary first of all to know the amount of true protein inside and outside the embryo on each day of incubation, and this was obtained by making various corrections; thus from the total nitrogen of the embryo were subtracted (*a*) the lipoid nitrogen, calculated from the work of Plimmer & Scott, and of Masai & Fukutomi, (*b*) the purine nitrogen, calculated from the work of LeBreton & Schaeffer, and (*c*) the water-soluble non-protein nitrogen, taken from the work of Needham. For the total protein nitrogen outside the embryo, the figures of Sakuragi were used, after being corrected for the use of trichloracetic acid instead of acetic acid and heat. The way these data were used is shown in Table 111. Columns 2, 3, and 4 concern the embryo and need no special remark.

[1] A most interesting sidelight on the nature of yolk-absorption is given by the work of Newman on hybrids of the minnows *Fundulus majalis* and *Fundulus heteroclitus*. The former fish lays eggs 2·7 mm. in diameter, the latter 2 mm. An *F. majalis* ♀ × *F. heteroclitus* ♂ cross gives after about a month a hybrid embryo resembling the paternal species, and too small for its yolk, i.e. unable to assimilate it. Although in the presence of excess food, its absorption-mechanisms seem incapable of dealing with it and the hybrid, sinking to the bottom, dies before it can hatch. The opposite hybrid (where the egg is small) hatches small, unable to reach the large size of its paternal origin, but is quite viable (cf. the genetic differences in avian yolk-absorption-rate mentioned on p. 940).

Table III. *Protein absorption of the chick embryo.*

| Interdiurnal period | Protein nitrogen increments in milligrams nitrogen stored by the embryo each day | Milligrams protein nitrogen combusted each day | Milligrams protein nitrogen absorbed each day, Col. 2 + Col. 3 | Milligrams true protein nitrogen in the whole egg minus lipoid nitrogen outside the embryo, for the interdiurnal periods | Milligrams true protein nitrogen in the embryo for the interdiurnal periods | Milligrams true protein nitrogen outside the embryo, Col. 5 − Col. 6 | Milligrams lipoid and purine nitrogen inside the embryo for the interdiurnal periods | Col. 7 − Col. 8 | Milligrams protein nitrogen absorbed each day in percentages of what remains to be absorbed | Weights of embryos for interdiurnal periods (grm.) (Murray and Needham) | | Absorption curve | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Wet | Dry | For wet weight | For dry weight |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 0–1 | — | — | — | 1001·2 | — | 1001·2 | — | — | — | — | — | — | — |
| 1–2 | — | — | — | 991·0 | — | 991·0 | — | — | — | — | — | — | — |
| 2–3 | — | — | — | 984·0 | — | 984·0 | — | — | — | — | — | — | — |
| 3–4 | 0·401 | — | 0·401 | 977·2 | 0·354 | 976·8 | 0·0297 | 976·80 | 0·0410 | 0·057 | 0·00308 | 71·93 | 1303 |
| 4–5 | 0·545 | 0·0023 | 0·547 | 968·6 | 0·827 | 967·8 | 0·083 | 967·70 | 0·055 | 0·165 | 0·00882 | 34·24 | 640 |
| 5–6 | 0·96 | 0·0026 | 0·963 | 961·1 | 1·55 | 959·6 | 0·218 | 959·38 | 0·095 | 0·322 | 0·01767 | 29·44 | 535 |
| 6–7 | 1·76 | 0·0099 | 1·77 | 950·8 | 2·89 | 947·9 | 0·425 | 947·47 | 0·187 | 0·579 | 0·0333 | 32·29 | 561 |
| 7–8 | 3·07 | 0·08 | 3·15 | 940·8 | 5·30 | 935·5 | 0·619 | 934·88 | 0·337 | 0·962 | 0·0584 | 35·02 | 577 |
| 8–9 | 4·46 | 0·18 | 4·64 | 931·4 | 9·07 | 922·3 | 0·788 | 921·51 | 0·503 | 1·503 | 0·09595 | 33·48 | 524 |
| 9–10 | 6·46 | 0·25 | 6·71 | 921·0 | 14·53 | 906·5 | 1·089 | 905·4 | 0·741 | 2·239 | 0·1522 | 32·05 | 486 |
| 10–11 | 10·12 | 0·34 | 10·46 | 911·4 | 22·82 | 888·6 | 1·524 | 887·1 | 1·179 | 3·205 | 0·2375 | 36·76 | 496 |
| 11–12 | 15·63 | 0·44 | 16·07 | 900·6 | 35·69 | 864·9 | 2·35 | 862·5 | 1·863 | 4·427 | 0·3691 | 42·09 | 505 |
| 12–13 | 23·71 | 0·55 | 24·26 | 890·2 | 55·36 | 834·8 | 3·797 | 831·0 | 2·919 | 5·972 | 0·5700 | 48·88 | 512 |
| 13–14 | 42·12 | 0·68 | 42·80 | 878·3 | 88·28 | 790·0 | 5·527 | 784·5 | 5·455 | 7·906 | 0·8947 | 69·01 | 610 |
| 14–15 | 60·49 | 0·83 | 61·32 | 866·7 | 139·58 | 727·1 | 7·45 | 719·65 | 8·521 | 10·217 | 1·3865 | 83·39 | 615 |
| 15–16 | 70·84 | 0·96 | 71·80 | 857·0 | 205·25 | 651·7 | 8·89 | 642·8 | 11·180 | 12·925 | 2·0170 | 86·50 | 554 |
| 16–17 | 60·48 | 1·17 | 61·65 | 844·4 | 275·91 | 568·5 | 10·82 | 557·68 | 11·060 | 16·170 | 2·7250 | 68·40 | 406 |
| 17–18 | 64·46 | 1·41 | 65·87 | 828·7 | 343·38 | 485·3 | 13·33 | 472·0 | 13·960 | 19·990 | 3·4885 | 69·81 | 400 |
| 18–19 | 54·86 | 1·93 | 56·79 | 812·7 | 403·04 | 409·7 | 15·34 | 394·4 | 14·400 | 24·350 | 4·3030 | 59·14 | 335 |
| 19–20 | 47·33 | 2·16 | 49·49 | 791·7 | 454·13 | 337·6 | 17·68 | 319·9 | 15·47 | 29·835 | 5·1595 | 51·87 | 299 |

Cols. 5 to 9 deal with the egg as a whole. Col. 5 gives the apparent protein nitrogen in the whole egg for the intermediate periods; it is obtained by considering the period "5th-to-6th-day", for instance, as if it were 5·5 days, and taking the average of the values for the 5th and 6th days. Col. 6 performs an exactly similar service for the true protein nitrogen in the embryo. It will be clear that, since Col. 5 is for the whole egg, the subtraction of Col. 6 from it will give figures for the remainder, the non-embryonic part of the egg. The result of doing this is shown in Col. 7, which represents the protein nitrogen in the remainder of the egg for the intermediate periods, corrected for the lipoid nitrogen outside the embryo, but still including the false protein nitrogen inside the embryo. Cols. 8 and 9 perform this final adjustment. Col. 8 shows the lipoid and purine nitrogen inside the embryo, calculated for the intermediate periods, and Col. 9 the true protein nitrogen in the remainder of the egg at any given moment during development.

Cols. 4 and 9 are now the ones on which attention must be focused. If Col. 4 is expressed as a percentage of Col. 9, we shall be finding what 100 mgm. of true protein nitrogen outside the embryo hand over during each interdiurnal period to the embryo. We shall have the milligrams absorbed each day in percentage of what each day remains to be absorbed.

It is clear from a summary inspection of the figures in Col. 10 that this value is not very illuminating. It only shows the gradual increase in size of the embryo. But if now this value is expressed as percentage of wet and dry weight, we shall be calculating what 100 mgm. of protein nitrogen hand over to 100 grams of embryo throughout development, and we shall be able to observe the varying intensities of the progress. Cols. 11 and 12 simply give the weight data of Murray and Needham calculated for the interdiurnal periods. Cols. 13 and 14 give the final results.

They are shown graphically in Fig. 250. It is seen that the absorption of protein, in the first few days of development very rapid, falls off exceedingly between the 6th and 10th days, to rise again, however, to a high peak on the 15th or 16th day. After that point it again falls to about its previous level. The most interesting thing to notice is that, as far as protein is concerned, there is no correlation whatever between absorption and combustion. The peak of protein combustion (see p. 993), which occurs between the 8th and the 9th

days, comes just in the trough of protein absorption. The two processes seem to be entirely distinct, as is clearly shown by the broken line representing protein combustion in Fig. 250. The 15th day peak corresponds remarkably well with the fact that about that time the vascular bag or "avian placenta" of Duval is formed by the fusing ends of the allantois. The accelerated absorption of protein from the egg-white shows itself as clearly in Fig. 250 as does the accelerated diminution of bulk of egg-white in Fig. 199. It is also interesting to note the effect of relating absorption to dry and to wet weight. The increasing dryness of the embryo has the result of minimising both the trough at the 7th day and the peak at the 15th.

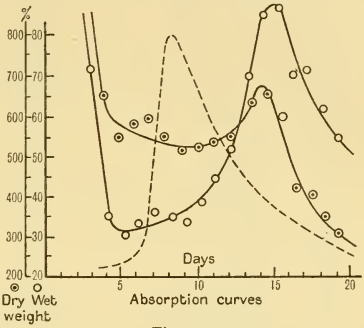



Fig. 250.

The process by which a knowledge of the absorption curve for fat was derived differed in no way from what has already been seen in the case of protein, except that it was less complex, fewer corrections to the basic values being necessary. Unfortunately, the resulting curves cannot claim the same degree of accuracy as may be accorded to those for protein, for an at present unresolved discrepancy exists in the literature between the measurements of egg-fat. As will be seen in Section 8·4, from the 7th to the 14th day the fat lost, as determined by the averaged chemical analyses, is considerably in excess of that lost as determined from the carbon dioxide output, even assuming that all the carbon dioxide was derived from fat, which is not true. But it is probable that this error, which is certainly real and of some theoretical importance, does not upset the general shape of absorption curves calculated disregarding it. When its nature is cleared up, the fat absorption curve may have to be revised.

In Table 112, the various stages of the calculation are set out. Columns 1 to 4 need no comment. In Cols. 5, 6 and 7 are shown, first, the fat in milligrams per whole egg, calculated for the interdiurnal periods from the experimental data of Eaves; Idzumi; Murray; and Sakuragi; secondly, the fat in the embryo calculated for the same

Table 112. *Fat absorption of the chick embryo.*

| Inter-diurnal period | Fat increment. Milligrams fat stored by embryo each day | Milligrams fat combusted each day | Col. 2 + Col. 3. Milligrams fat absorbed each day | Milligrams fat in whole egg for the inter-diurnal periods | Milligrams fat in embryo for the inter-diurnal periods | Col. 5 − Col. 6 | Milligrams of fat absorbed each day in percentages of what remains to be absorbed | Absorption curve | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | For wet weight | For dry weight |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 0–1 | — | — | — | — | — | — | — | — | — |
| 1–2 | — | — | — | — | — | — | — | — | — |
| 2–3 | — | — | — | — | — | — | — | — | — |
| 3–4 | 0·47 | 0 | 0·47 | 5600 | 0·435 | 5600 | 0·0084 | 9·883 | 210·0 |
| 4–5 | 1·18 | 0 | 1·18 | 5600 | 1·26 | 5598·7 | 0·0211 | 12·790 | 239·1 |
| 5–6 | 1·64 | 0 | 1·64 | 5600 | 2·67 | 5597·3 | 0·0293 | 9·100 | 165·5 |
| 6–7 | 3·04 | 0 | 3·04 | 5600 | 5·01 | 5595·0 | 0·0545 | 9·413 | 163·7 |
| 7–8 | 4·98 | 20 | 24·98 | 5590 | 9·02 | 5581·0 | 0·4476 | 46·52 | 766·5 |
| 8–9 | 7·50 | 50 | 57·50 | 5555 | 15·26 | 5539·7 | 1·038 | 69·06 | 1081·0 |
| 9–10 | 11·73 | 80 | 91·73 | 5490 | 24·87 | 5465·0 | 1·678 | 74·95 | 1103·0 |
| 10–11 | 18·35 | 94 | 112·35 | 5400 | 39·91 | 5360·0 | 2·095 | 65·37 | 882·2 |
| 11–12 | 29·56 | 105 | 134·56 | 5300 | 63·87 | 5236·0 | 2·571 | 58·08 | 696·6 |
| 12–13 | 47·05 | 116 | 163·05 | 5190 | 102·17 | 5087·8 | 3·204 | 53·64 | 562·0 |
| 13–14 | 82·00 | 127 | 209·00 | 5070 | 166·70 | 4903·0 | 4·262 | 53·91 | 476·4 |
| 14–15 | 125·55 | 145 | 270·50 | 4935 | 270·47 | 4664·5 | 5·800 | 56·75 | 418·4 |
| 15–16 | 164·75 | 168 | 332·75 | 4765 | 415·62 | 4349·4 | 7·649 | 59·20 | 379·3 |
| 16–17 | 203·50 | 205 | 408·50 | 4585 | 599·70 | 3985·0 | 10·250 | 63·26 | 375·3 |
| 17–18 | 278·05 | 250 | 528·05 | 4375 | 840·52 | 3534·5 | 14·940 | 74·73 | 428·4 |
| 18–19 | 355·45 | 331 | 686·45 | 4080 | 1157·27 | 2922·7 | 23·480 | 96·43 | 545·6 |
| 19–20 | 429·00 | 460 | 889·00 | 3680 | 1549·50 | 2130·5 | 41·740 | 139·90 | 809·0 |

lapses of time, and, finally, the difference between the two, in other words, the milligrams of fat present in the non-embryonic part of the egg in the intermediate periods. In Col. 8 is found the milligrams absorbed each day expressed in percentage of what at that day remains to be absorbed. This is the column representing the amount of fat handed over to the embryo out of 100 mgm. of external fat between each two days. Cols. 9 and 10 express the same value only related to 100 gm. of embryo, wet and dry. When Cols. 9 and 10 are plotted upon a graph, very interesting curves are seen. Fig. 251 shows that the absorption curve for fat rises and falls in much the same way as that for protein. It possesses a peak about the 10th day, and another rapid rise about the 18th[1]. What is at once noticeable is that these periods do not synchronise with the period of pre-eminent fat combustion. The 15th day is the centre of the period at which the respiratory quotient is 0·73 (Bohr & Hasselbalch), yet at that very day there is a distinct trough in the curve of fat absorption. We see, then, that, as with protein, so with fat, there is no chronological relation between combustion and absorption.

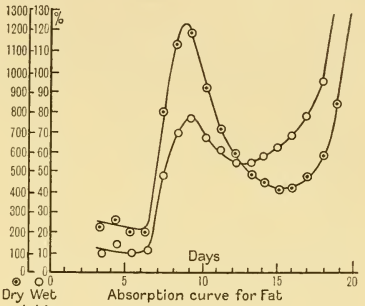



Fig. 251.

Secondly, it may be pointed out that the observation of Gage & Gage, that Sudan III eggs do not give coloured embryos till the middle of development, fits in well with the absorption curve now found. Up to the 8th day absorption of fat goes on very slowly.

The third point of interest is this. Although the curve for fat rises and falls in much the same way as that for protein, it does it at quite different times; it does not resonate with it; protein peaks correspond to fat troughs and *vice versâ*. This is well seen in Fig. 252, which shows the averages between wet and dry weights in each case. It should

[1] The only other absorption-curve which has been studied is that for lead (Bishop). It shows a marked trough at the 15th day, corresponding with Fig. 251. This is interesting because Bishop concluded on quite other grounds that lead is present in yolk almost wholly in combination with lecithin (e.g. 99% of it is ether-soluble). Lead lecithin and lead oleate can, indeed, be isolated from yolk.

be well understood that Fig. 252, as far as absolute values go, is meaningless: it would represent the absorption of embryos 50 per cent. less rich in water than they really are: but it demonstrates more clearly the relationship of fat and protein. In the last stages of development, "it is as if the protein were displaced by fat", says Murray, speaking of fat storage, and this is just what Riddle found in his studies of the yolk in pigeons' eggs. At the end of development there was a marked preferential absorption of fat. Thus from the 1st to the 6th day very little fat is being absorbed, but a great deal of protein, from the 6th to the 12th day exactly the reverse holds, from the 12th to the 17th day the protein again takes the prominence, while after that time the fat once more overcomes the protein. The rhythms of the two absorption curves are entirely distinct.

At first sight, it would not seem easy to estimate the significance of this. One way of expressing it would be to say that the cells of the blastodermal blood-vessel walls which collect the nourishment from the yolk and the white have periods at the ends and beginnings of which the properties of their membranes alter. At one time they will admit fat-soluble substances in predominance, at another time water-soluble substances. Put in this way, the phenomenon immediately reminds us of the state of affairs seen by many observers in the egg of the sea-urchin where there are periods of permeability to water-soluble substances, and other periods of permeability to fat-soluble substances. At one point the resistance to alcohol-chloroform-ether cytolysis is remarkably high, at another it is extremely low. A similar curve can be prepared for a water-soluble substance, such as potassium cyanide, and its summits are seen to correspond to the troughs of the previous one. That rhythms of permeability to fat-soluble and water-soluble substances should be present in the single developing egg-cell of the lower animals, and should then appear again in the complicated avian organism during its ontogenesis is certainly possible, and, if real, of considerable

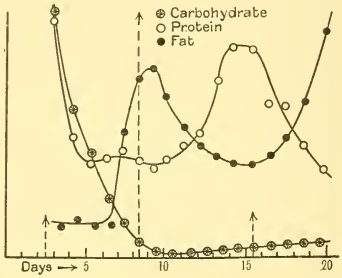



Fig. 252. The reason for the ungraduated ordinate is explained in the text.

interest. It must be stated, however, that the rhythms of permeability and susceptibility during the cleavage of echinoderm eggs are not regarded as significant by some investigators, who ascribe them to purely mechanical causes associated with the changing shape of the embryo during the cleavage stages. An account of them will be found in the Section on resistance and susceptibility.

How does the absorption intensity of carbohydrate vary during the development of the chick? The study of the carbohydrate metabolism of the hen's egg which I made in 1927 provided the data which were requisite for the answering of this question. Exactly the same procedure which had been previously applied to protein and fat was applied to carbohydrate; the calculations appear in Table 113, and the resulting curve in Fig. 252 alongside the curves for protein and for fat. The difficulty here was to assess the amount of glucose combusted during each period. This can evidently be no more than a rough approximation, for no accurate data exist on carbohydrate combustion, nor is it easy to see how they could be obtained, since sugar is burned completely away to carbon dioxide and water, leaving no end products whose concentration can be measured. Fiske & Boyden pointed out that the combustion of 100 mgm. of glucose would produce 75 c.c. of carbon dioxide, whereas in the first five days the embryo only produces 10 c.c. and Col. 3 was constructed bearing this in mind. All the carbohydrate lost cannot have been combusted. Col. 4 gives the sum of Cols. 2 and 3, i.e. the amount absorbed in each period, and Col. 5 shows the amount remaining outside the embryo. In Col. 6, Col. 4 is expressed in percentage of Col. 5; in other words, this gives the amount absorbed each day in percentage of the amount remaining to be absorbed. Cols. 7 and 8 give the weights of the embryo, dry and wet, calculated from Murray's data and some of mine. Cols. 9 and 10 show the intensity of absorption of carbohydrate calculated for wet and dry weight.

The two questions which this curve answered were (*a*) whether there was any relation of simultaneity between the absorption and combustion of carbohydrate, and (*b*) whether there was any likeness between the absorption curves for protein and carbohydrate, for, if so, the conception of rhythmic permeability changes on the part of the cells of the blastodermal blood-vessels would receive support.

The period of predominance of carbohydrate combustion is believed to be in the first week of development, and from Fig. 252 it

Table 113. *Carbohydrate absorption of the chick embryo.*

| Date | Increment in total carbohydrate (mgm.) | Milligrams total carbohydrate combusted | Cols. 2+3. Milligrams total carbohydrate absorbed | Milligrams outside the embryo | Col. 4 as % of Col. 5 | Weights of embryo for interdiurnal periods in milligrams | | Absorption curve | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Wet | Dry | Wet | Dry |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 0–1 | — | 0 | 0 | 315 | 0 | — | — | — | — |
| 1–2 | — | 1 | 1 | 277 | 0·36 | — | — | — | — |
| 2–3 | — | 2 | 2 | 240 | 0·83 | — | — | — | — |
| 3–4 | — | 3 | 3 | 208 | 1·44 | 57 | 3·08 | 2·53 | 46·8 |
| 4–5 | 0·164 | 4 | 4·2 | 181 | 2·32 | 165 | 8·82 | 1·41 | 26·3 |
| 5–6 | 0·206 | 5 | 5·2 | 160 | 3·25 | 322 | 17·67 | 1·01 | 18·4 |
| 6–7 | 0·302 | 4 | 4·3 | 146 | 2·95 | 579 | 33·3 | 0·51 | 8·9 |
| 7–8 | 0·472 | 3 | 3·5 | 142·5 | 2·46 | 962 | 58·4 | 0·26 | 4·2 |
| 8–9 | 0·770 | 2 | 2·8 | 166 | 1·69 | 1,503 | 95·9 | 0·11 | 1·8 |
| 9–10 | 1·09 | 1 | 2·1 | 210 | 1·00 | 2,239 | 152·2 | 0·045 | 0·7 |
| 10–11 | 1·52 | 0 | 1·5 | 229·5 | 0·65 | 3,205 | 237·5 | 0·02 | 0·3 |
| 11–12 | 2·88 | 0 | 2·9 | 221·7 | 1·31 | 4,427 | 369·1 | 0·029 | 0·35 |
| 12–13 | 3·66 | 0 | 3·7 | 203·5 | 1·82 | 5,472 | 570 | 0·033 | 0·32 |
| 13–14 | 6·61 | 0 | 6·6 | 184 | 3·58 | 7,906 | 895 | 0·045 | 0·4 |
| 14–15 | 7·96 | 0 | 8·0 | 169·5 | 4·72 | 10,210 | 1386 | 0·046 | 0·34 |
| 15–16 | 11·00 | 0 | 11·0 | 151·5 | 7·26 | 12,920 | 2017 | 0·056 | 0·36 |
| 16–17 | 15·30 | 0 | 15·3 | 133·5 | 11·50 | 16,170 | 2725 | 0·071 | 0·42 |
| 17–18 | 16·4 | 0 | 16·4 | 120 | 13·70 | 19,990 | 3488 | 0·068 | 0·37 |
| 18–19 | 18·9 | 0 | 19·0 | 100 | 19·00 | 24,350 | 4303 | 0·078 | 0·44 |
| 19–20 | 19·8 | 0 | 20·0 | 79 | 25·30 | 29,830 | 5159 | 0·084 | 0·49 |

may be seen that the absorption intensity is then at its highest. In this way carbohydrate differs from protein and fat.

During the first 6 days the absorption of carbohydrate and protein (both "water-soluble substances") is very high, while that of fat is very low. From the 6th to the 13th days the intensity of absorption of fat is high, and protein and carbohydrate are low. From the 13th to the 17th days the absorption of fat again drops, and that of protein rises, but carbohydrate does not accompany it; on the contrary, it remains very low, though moving slowly in an upward direction. At the 18th day there is another sharp cross-over between protein and fat which is not shared by carbohydrate.

The behaviour of carbohydrate gives, therefore, some support to the theory that changing types of permeability in the yolk-sac cells are responsible for these effects. In the earlier periods when the absolute amounts of carbohydrate being absorbed are comparable with the absolute amounts of fat and protein being absorbed, the protein-carbohydrate curve does tend to be the reciprocal of the fat curve, but later on the carbohydrate drops out of the relationship, and pursues an uneventful course of its own. Thus on the 6th day the embryo absorbs 5 mgm. of carbohydrate, 6 of protein and 2 of fat, while on the 16th day it absorbs 11 of carbohydrate, 449 of protein and 333 of fat. During the period, then, when the absorption of carbohydrate is at all equivalent to that of the other food-stuffs the relations predicted by the hypothesis hold in practice.

It will be worth while to compare carefully the curves for absorption intensity in the chick embryo, with those for its constitution, for the correlation between constitution and absorption is quite close. When the intensity of absorption of carbohydrate is at its highest, then there is more carbohydrate in the embryo than at any other time. When the intensity of absorption of protein is passing its second peak the constitution curve has also its peak. When the intensity of absorption of fat has risen to its highest level the amount of fat in the embryo has done so too.

On the other hand, there are two points where the correlation is not obvious. The first peak on the fat absorption curve has no corresponding inflection on its constitution curve, and the initially high absorption intensity of protein has nothing to correspond with it. There is, of course, no *a priori* reason to expect close agreement between the peaks on the absorption and constitution curves.

When one shows that a certain amount of one kind of building-stone has entered the embryo and the other shows that it is not to be found there in the form in which it went in, the inference may be drawn that it has been changed into some other material of architectural or energetical value. Now not all the fat entering the embryo between the 8th and 11th days appears there immediately afterwards as fat. One cannot help being reminded of the coincident transformation of fat into carbohydrate, which will be discussed below in Section 8·4. At that time some 40 mgm. of carbohydrate rather suddenly appear from somewhere, and at the same time there is a quantity of missing fat of about the same order. That these transformations appear to take place in the extra-embryonic part of the egg is no objection to the view that they really take place in the embryo, for the circulation in the egg is fairly efficient at that time, and could readily bring the reactants in this change to and from the embryo. In fact, just as we are accustomed to assume that absorption must precede combustion, so it would be logical to assume that absorption must precede fat-carbohydrate transference. There would then be no reason why the products of this reaction should remain in the embryo. Although the absolute amount of substance thus transformed is small enough yet relatively to the size and constitution of the embryo at the time when it takes place, it would account for the peak in the absorption curve. If these arguments are sound, then we must picture an increased passage of fat into the embryo towards the mid-point of development, having for its goal the formation of some new carbohydrate. This will then return to the yolk, and in a short time give rise partly to glycogen in the transitory liver and partly to some other form of combined sugar.

It will have been noticed that, though the peak in the protein constitution curve corrected for feather protein shows some correspondence with the protein combustion curve (11 days : 8·5 days), it would be rather more fitting when comparing the constitution curve with the absorption curve to use the uncorrected values. When this is done, there is revealed an exact correspondence (16 days : 15 days).

The method of calculation which has proved so successful in comparing relative absorption intensities in the case of the chick embryo unfortunately cannot be applied to mammals, for we do not know, and at present have no means of knowing, what the active mass of

the foodstuff provided by the maternal organism amounts to. We cannot, therefore, enquire how many grams 100 gm. of raw material hands over to 100 gm. of formed embryo in a given time. Consequently the data we have on the absorption of material by the mammalian embryo are fragmentary, and are almost entirely concerned with the problem of how much the mother on a given diet can afford to store away in the developing embryo.

Thus Magnus-Levy calculated that the average daily deposition in the human foetus for the last hundred days of its development represents not more than 3·0 gm. of protein, 3·5 gm. of fat, and about 0·7 gm. of ash. His table was as follows:

| | Weight in gm. | | Nitrogen | Fat | Ash |
|---|---|---|---|---|---|
| | Wet | Dry | (gm.) | (gm.) | (gm.) |
| 7 months embryo ... | 950 | 155 | 16 | 26 | 26 |
| Embryo at term ... | 3200 | 925 | 62·5 | 350 | 92·5 |
| Increase in 100 days ... | 2250 | 770 | 46·5 | 324 | 66·5 |
| Increase per day ... | 22·5 | 7·7 | 0·46* | 3·24 | 0·665 |

* I.e. of protein 3·0.

It is not possible to enter here into the question of whether the absorption of raw materials by the mammalian embryo entails loss from the maternal tissues or not. The question is a very complex one, and reference should be made to the reviews of Magnus-Levy; Feldman; Hoffström; Eckles; Harding; and Murlin, and to an anonymous one which, although now some twenty years old, is worth consulting. Mention may be made, however, of the interesting case studied by Rubner & Langstein, where a 7-months embryo was born, and was available for the study of the absorption and retention of the food. The infant weighed 2050 gm. at birth, but 8 days afterwards its weight had diminished to 1900 gm., though after that it began to increase by about 28 gm. a day. When the experiments began it weighed 2360 gm. During the next 11 days it retained 50 per cent. of the nitrogen in its milk, though at this time, which would have corresponded to the 8th month of pregnancy, the addition of protein to the child amounted to only a half of that computed by Hoffström for the foetus of the same age. The diet contained 126 Cal. per kilo body-weight, of which 73 were used for heat production (973 Cal. per sq. metre per day) and 53 were deposited in the infant. Altogether 42 per cent. of the Calories taken in with the food were retained for growth.

It is interesting to enquire what is the absorption rate of the embryo at the different stages of its development In the case of the mammal this is again impossible, for we know nothing quantitative about the substances burned by it. Its storage rate can, of course, easily be calculated from the figures of Michel or Fehling, thus:

| Months | Weekly percentage increment of nitrogen storage in the human embryo |
|---|---|
| 5 | 0·22 |
| 9 | 0·20 |

but such a calculation tells us nothing more than that the percentage growth-rate is declining, a fact already well known. The true absorption rate has only been calculated for the chick embryo. Murray obtained it in 1926 from his determinations of carbon dioxide excreted and oxygen taken in by the chick embryo from the 5th day of incubation onwards by simply adding the rate of storage in terms of weight (see p. 384) to the rate of respiratory exchange, likewise in terms of weight measured by oxygen usage. His values are shown in Fig. 253, where the rate of dry solid absorption per gram of embryo (dry) per day is plotted against the age. Evidently the curve falls rapidly. But the use of the equation:

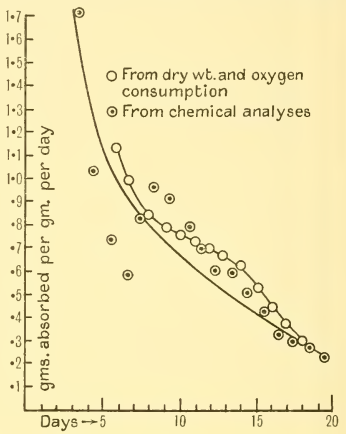


Fig. 253.

Solid stored + solid burnt = solid absorbed

can also be demonstrated in another way, namely by adding together the results of all the chemical analyses of protein, fat and carbohydrate and ash. Of these we have very reliable figures for the amounts of carbohydrate, protein and fat stored, and for the amount of protein combusted, but the amounts of fat and carbohydrate combusted have never been directly measured, and must therefore be approximated. Nevertheless it was of interest to calculate the theoretical absorption

curve from these biochemical data, and see how well they agreed with the curve obtained by Murray. The curve so resulting is placed beside Murray's in Fig. 253. His smoothed curve is S-shaped, but that calculated from the chemical analyses tends rather to be uninflected. Considering the many operations involved in the establishing of the chemical curve the agreement is good, but it is not possible to decide which curve is the more reliable.

The consumption of food, as Murray pointed out, is enormous in the early periods. On the 6th day, for instance, the embryo absorbs over its own mass of dry solid, which would be equivalent to an adult man eating about 150 pounds of food per day. During the time between the 6th and the 18th days of incubation, this rate falls to about a quarter of its original value. According to Lotka, a mature meadow-lark consumes about 6·6 of its own weight in one day, so that the fall in absorption rate must continue for a considerable time after hatching.

A few words may be included at this point about the routes of absorption of material by the embryo, for the subject is not wholly of morphological importance. Wislocki injected trypan blue into the air-chamber, yolk-sac, allantoic and amniotic sacs, and allantoic mesoblast at 11 days' incubation. On opening 2 days later, no staining of embryo or membranes was observed after injections into the air-chamber, but the trypan blue in the yolk was absorbed vigorously by the epithelial cells lining the interior of the yolk-sac. Eventually the dye penetrated the basement membrane on which the endoderm rests, and reached its final destination in groups of cells surrounding the rich venous network in the yolk-sac wall. It did not pass into the vessels and so to the embryo, but it well illustrated how other substances would do so. In spite of the connection between the yolk-sac and the intestinal lumen through the vitelline duct no trypan blue passed into the embryo by that means. From the allantoic cavity no absorption of trypan blue occurred. From the amniotic cavity there was some passage of the dye into the intestines but no other absorption. Hammar next reported a few generally similar results with neutral red & cresyl blue, and Hanan later continued these studies with trypan blue and methylene blue. Making injections into the air-space and examining the egg at varying periods subsequently, he found that as a rule the stain appeared in the egg-white, the allantoic liquid and membrane, and in the embryo,

especially the mesonephros—but not in the yolk, the amniotic liquid, the amniotic membrane or the lumen of the intestine, although if the egg was not opened for a week or so after injection, the dye would appear in the second group of structures also. The fact that trypan blue, although present in the egg-white at a time when a marked absorption of water is going on by the yolk, does not enter the yolk, throws a light on the properties of the vitelline vessels. Similar work was done by Latta & Busby[1]. Zaretzki also noted that methylene blue does not enter the yolk from the albumen. After the opening of the sero-amniotic duct about the 12th or 13th day of incubation, the amniotic liquid includes flakes of the protein from the albumen-sac, stained blue, and these enter the chick's intestine. Wislocki's failure to obtain entry of the dye into the egg from the air-space must have been due to the use of too small injections, for Hanan found that dye appears in the mesonephros about $1\frac{1}{2}$ hours after injection into the air-chamber.

### 6·10. Storage and Combustion: the Plastic Efficiency Coefficient

Absorption of raw materials for embryonic growth can be considered in several ways. Related to the embryonic substance already formed, it gives absorption rate; related to the embryonic substance already formed and to the raw material remaining, it gives absorption-intensity; related to the amount of substance being simultaneously combusted, it gives storage efficiency. We may now consider the last-named of these entities. The substrates of the absorption process divide perforce into two parts, one of which is stored and the other combusted as a source of energy.

The degree of efficiency with which the transference of yolk and albumen into flesh and blood is effected may most conveniently be expressed by an efficiency coefficient. This corresponds to the "Rendement materiel" of Henri, the "Coefficient d'utilisation" of Terroine & Wurmser, the "Coefficient économique" of Pfeffer, and the "Plastic equivalent" of Waterman. The best name for it would seem to be "Plastic efficiency coefficient" (P.E.C. for short), for this shows that it has nothing to do with energy content or expenditure, and explains that it is a measure of efficiency of transfer of matter. It may be described as the ratio:

Dry weight of embryo/Dry weight of absorbed solid,

[1] Fazzari, too, has studied the absorption of iodine after injection into the yolk.

and naturally shows the relative cost in grams of solid of building the embryo. The higher the efficiency coefficient, the smaller the amount of burnt substance in relation to stored substance.

Gray, in his memoir on the chemical embryology of the trout, already referred to, found that its average plastic efficiency coefficient (P.E.C.) was 0·63. He worked it out for the chick from Murray's data in a cumulative way, but a more instantaneous picture is given when it is calculated on a daily basis, as I showed in 1927. How expensive is it on each day of development to build what is built on that day? It is evident from Fig. 254 that both curves fall and then rise, and the lag in the cumulative one is not significant, for each day's point bears, as it were, in itself the effects of the previous days. The incremental P.E.C. shows the instantaneous change.

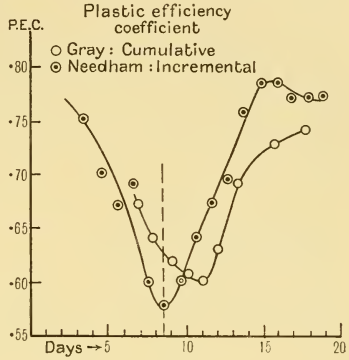


Fig. 254.

There must be some significance in the deep trough through which the curve passes between the 7th and 12th days. Evidently at that period development is most expensive; the amount of burnt substance is greater relatively to the amount of stored substance then than at any other time. This suggests a correlation with the intensity of protein combustion, which is indeed very exact—in fact, strikingly so, as may be seen from the vertical line in Fig. 254. The inference that we have here to deal with an effect of specific dynamic action is difficult to resist, but probably more than one factor is responsible.

The average P.E.C. for the whole of the chick's development is 0·68. It is interesting to enquire which of the food-stuffs contribute principally to this degree of efficiency. Knowing that fat is the chief food-stuff combusted, and that protein is the chief architectural material, it would be natural to predict that the most efficiently stored substance would be protein. The exact figures follow.

Table 114.

| | | Mgm. | P.E.C. | % of total food-stuff combusted |
|---|---|---|---|---|
| Carbohydrate | stored ... | 107 | 0·82 | 5·6 |
| | burnt ... | 25 | | |
| Protein | stored ... | 2986 | 0·98* | 3·02 |
| | burnt ... | 69 | | |
| Fat | stored ... | 1700 | 0·43 | 91·4 |
| | burnt ... | 2110 | | |
| Total solid stored ... | | 4793 | Av. 0·68 | |
| Dry weight of embryo at 19·5 days approx. | | 5000 | | |

* Fiske & Boyden by independent reasoning, arrived at 0·96 for this value, and Sznerovna's data give 0·92.

Out of 100 gm. of protein in its diet, then, the embryo can store away 98, out of 100 gm. of carbohydrate 82, but out of 100 gm. of fat only 43. In the case of animals such as the trout, which burn large amounts of protein, the "foodstuff P.E.C." will be very different.

The average P.E.C. can be calculated for a number of other organisms:

| Organism | P.E.C. | Investigators |
|---|---|---|
| Mould (*Aspergillus niger*) ... ... | 0·59 | Terroine & Wurmser |
| Silkworm (*Bombyx mori*) embryo ... | 0·59 | Farkas |
| Trout (*Salmo fario*) embryo ... ... | 0·63 | Gray |
| Frog (*Rana temporaria*) embryo ... | 0·58 | Fauré-Fremiet & Dragoiu |

The average efficiency of transfer of the material yolk and white into the material of the embryo seems, then, to be constant for a wide variety of species. But it is now generally recognised that these average figures give very little information about what is actually going on, and it is therefore necessary to enquire how the P.E.C. varies during the developmental process. We have no data for this in the case of any other animal than the chick, except Gray's work on the trout. The growth-rate of this embryo falls off during the later stages of its development, and as its metabolic rate (respiratory intensity, or maintenance intensity) was found by Gray to remain constant, then evidently its efficiency must get smaller as it grows. This relative constancy in the metabolic rate differentiates it sharply, of course, from the chick (see Figs. 126 and 143). Gray accordingly computed the P.E.C. for each successive half-gram of yolk, with the following results:

| | |
|---|---|
| 0·76 | 0·54 |
| 0·71 | 0·43 |
| 0·65 | 0·19 |

Hayes, on the other hand, found exactly the opposite on the Atlantic salmon; a rising instead of a falling P.E.C.; 0·36 before the 100th day and 0·52 afterwards. Obviously a further extension of our knowledge of plastic efficiency coefficients would be very desirable.

The effect of temperature upon the P.E.C. raises an interesting problem. For the mould, *Aspergillus niger*, Terroine & Wurmser in their classical paper, could find no difference, thus

| Temperature ° C. | P.E.C. |
|---|---|
| 22 | 0·44 |
| 29 | 0·43 |
| 36 | 0·43 |
| 38 | 0·44 |

They therefore concluded that although in a given time the amount of mycelium formed would be less at the lower temperature, the amount of material combusted would be correspondingly less. Combustion would always go parallel with storage. Rubner found much the same thing in the case of the growth of *Proteus vulgaris*, and Terroine & Wurmser were led to formulate the general rule "that the quota of material and energy utilised during a given piece of biological work, does not vary appreciably within the limits of temperature compatible with life, although the rate at which the work is done will, of course, vary greatly with temperature". In general this conclusion was supported by the experiments of Terroine, Bonnet & Joessel with germinating seeds (for further discussion of this work see Appendix IV)

| | Temperature ° C. | P.E.C. |
|---|---|---|
| Sorghum ... | 33 | 0·54 |
| | 17 | 0·77 |
| Lentil ... ... | 30 | 0·56 |
| | 18 | 0·57 |
| | 11 | 0·56 |
| Linseed ... | 21 | 0·89 |
| | 11 | 0·83 |
| Arachis ... | 30 | 0·85 |
| | 17 | 0·86 |

Next Barthélemy & Bonnet examined the development of frog embryos at various temperatures, and obtained for the P.E.C. the following results:

| | Temperature ° C. | P.E.C. |
|---|---|---|
| 1st series ... | 8 | 0·88 |
| | 10 | 0·51 |
| | 14 | 0·54 |
| | 21 | 0·49 |
| 2nd series ... | 8 | 0·85 |
| | 10 | 0·79 |
| | 14 | 0·84 |
| | 21 | 0·83 |

Clearly the second of these series was in agreement with the law of Terroine & Wurmser, but the first was not. In the first series the higher the temperature the lower the efficiency, i.e. the more the relative amount of combustions and the less the relative amount of storage (although the figures do not show a regular progression). Parallel experiments were made by Gray on the trout embryo, with the following results:

| | | Temperature ° C. | P.E.C. |
|---|---|---|---|
| 1st series | ... | 10 | 0·56 |
| | | 15 | 0·50 |
| 2nd series | ... | 5 | 0·49 |
| | | 9 | 0·43 |
| | | 17·5 | 0·34 |

Gray emphasised these figures, which in his opinion demonstrated that the combustion processes had a higher temperature coefficient than the storage ones, i.e. that development was most efficient at low temperatures, for then storage was carried on to the accompaniment of less combustion. As we have already seen, he used these data to support a particular theory of embryonic growth (see Section 2·6), but it is sure that the problem cannot yet be regarded as settled especially in view of the fact that many researches demonstrate the energetic efficiency to be uninfluenced by temperature (cf. the Section on energetics)[1].

The P.E.C. of embryonic growth would seem to be distinctly higher than that of post-embryonic growth. An example could be taken from the work of Farkas and Kellner on the silkworm. On the other hand, if only the early part of development in some organisms be considered the P.E.C. may be still higher. Thus Parnas & Krasinska calculated that as a hatched frog larva might be regarded as containing 44 per cent. dry solid, of which about 12 per cent. would be unused yolk, 32 per cent. would be the dry solid of the embryonic body. Now one frog's egg, according to their data, consumes 27 c.mm. oxygen from fertilisation to hatching, or 0·039

[1] The work of Wood furnishes a suggestion with regard to this discrepancy. He found that trout embryos reared at 7° and 12° gave a P.E.C. of 0·63, which was not far from Gray's figures for 10°. At 3°, however, the P.E.C. was 0·55. In Wood's view, constancy of P.E.C. only occurs within the optimum range of development, and at lower or higher temperatures the processes of combustion and storage are dislocated. But, paradoxically, Wood's final larval size was smaller the lower the temperature; exactly opposite to the results of Gray.

mgm. which would correspond at the outside to 0·054 mgm. carbon dioxide or 0·0146 mgm. carbon. And the hatched larva weighing 2 mgm. has about 0·6 mgm. of protein in it, or 0·3 mg. carbon, so that only about 5 per cent. of the carbon absorbed was burned. The P.E.C. was therefore about 95 per cent. or 0·95 but of course hatching in amphibia is not the end of development, and before all the remaining yolk is used up the efficiency will have fallen to the usual 60 per cent. or 0·6.

### 6·11. Metabolism of the Avian Spare Yolk

This will be a convenient place in which to give an account of the yolk which is still available for the chick at the time of hatching. In the succeeding sections of the book, the metabolism of the yolk and white during the pre-natal stages within the egg will be fully unravelled, in so far as this is possible in the present state of our knowledge, and the data will be found in their appropriate places according to the section-headings. But the "spare yolk", as it is called, has been investigated so little, and its significance so seldom discussed, that it may as well be dealt with here. The most complete study of it is that of Iljin, though it was first attacked by Virchow. Iljin allowed newly hatched chicks to go without food for a number of days and from time to time weighed the bodies and the unutilised spare yolk. His data, which are plotted in Fig. 255 show that although the dry weight of the yolk diminishes enormously during the first few days after hatching, the dry weight of the chick remains practically constant. This yields an important result, namely, that the chick has absorbed all that it required for architectural purposes from the yolk before it hatches, and that after that moment, the "spare yolk" plays an entirely nutritive part, functioning mainly if not entirely as combustible material. "The chicken lives on its yolk", said Iljin, "and does not destroy the organised parts of its body, which have only just been formed." Now as will appear in later sections, and largely owing to the work of Riddle, we know that during the last week of incubation, the chick absorbs material from the yolk in varying intensity, lipoids being assimilated more rapidly than fats, and neutral fat more rapidly than proteins. It looks, therefore, as if the yolk at the time of hatching is much more preponderantly composed of protein than it was at the beginning of incubation, and as we know from Iljin's experiments

that after hatching it is entirely used for purposes of combustion, we cannot avoid the conclusion that the catabolism of fatty acids which was so prominent a feature of the pre-natal period, gives place to combustions in which protein plays a greater part, as soon as hatching is completed. Nor is it unwarranted to see in this arrangement a state of affairs quite in accord with the fact that disposal of nitrogenous waste-products is not easy before hatching and *is* easy afterwards. For further discussion on this subject see Section 9·15.

Referring again to Fig. 255 it will be noticed that if the chicks were given food a day or two after hatching, there was hardly any diminution in the dry weight of the spare yolk, as is indicated by the dotted line. No doubt it takes a long time to disappear completely if the circumstances of post-natal life are favourable to survival. "Extracting the spare yolk from the stomach", said Iljin, "we saw that it is included in a special tunicle like a sack. This sack opens into the thin gut by means of a special connection, the inside surface of which is corrugated, like the bile-duct of man. The wall of the gut makes a wrinkle above the opening which covers it like a valve, so that the contents of the gut cannot enter the duct during the peristaltic movements."

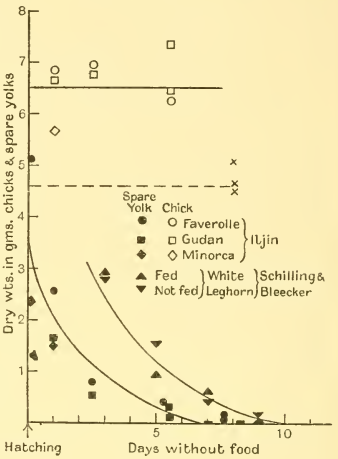



Fig. 255.

That the spare yolk forms a considerable part by weight[1] of the newly hatched chick is clear, in any case, from Iljin's data, which show from 13·6 to 27·8 per cent. of the wet weight and from 23·1 to 52·2 per cent. of the dry weight.

[1] The exact degree to which yolk is assimilated prior to hatching has been investigated by Jull & Heywang. There is no sex difference in the amount of spare yolk, but considerable variation according to the hen laying the eggs. The average amount of spare yolk is 40·78 % of the original yolk, but it may vary from 32·14 to 46·88 %, and as the eggs from individual hens are fairly uniform in this respect, the phenomenon appears to be genetic in origin (cf. the teleostean hybrids described on p. 920).

Later work by Schilling & Bleecker afforded further data about the absorption of the spare yolk by the hatched chick. They observed sometimes a curious failure to absorb it; thus in one instance an amount of 4·8 gm. was found when there should, according to the normal curve, only have been 0·021 gm., and in another case 2·95 instead of 0·62. Their conclusions differed a good deal from Iljin's, for they found no difference in rate of absorption between well-fed and ill-fed chicks[1], nor did the amount of yolk unabsorbed seem to bear any relation to the growth-rate of the individual.

Romenski, a student of Iljin's, studied the question from another angle, that of nitrogen utilisation, and drew up, as the result of his observations, the following table:

| | Gm. |
|---|---|
| Average weight of the chick at hatching ... ... ... | 37·60 |
| Average weight of chick minus its spare yolk ... ... ... | 30·00 |
| Nitrogen content of chick minus spare yolk (i.e. 57·97 % of the original store of nitrogen in the egg) ... ... ... ... | 0·499 |
| Average weight of the spare yolk ... ... ... ... | 6·951 |
| Nitrogen content of the spare yolk (i.e. 32·32 % of the original store of nitrogen in the egg) ... ... ... ... ... | 0·271 |
| Average weight of shell, membranes, and excreta ... ... | 0·885 |
| Nitrogen content of shell, membranes, and excreta (i.e. 9·55 % of the original store of nitrogen in the egg) ... ... ... | 0·058 |

He then subjected the hatched chicks to 36 hours' starvation or, more strictly speaking, he allowed them no other nourishment than that contained in their yolk-sacs. The results of similar estimations at the end of that time were as follows:

| | Gm. |
|---|---|
| Average weight of chick after 36 hours (minus its spare yolk)... | 33·60 |
| Nitrogen content of chick body ... ... ... ... ... | 0·534 |
| Average weight of spare yolk after 36 hours ... ... ... | 3·65 |
| Nitrogen content of spare yolk after 36 hours ... ... ... | 0·111 |

From these facts it is clear that during the post-hatching period the yolk lost 160 mgm. of nitrogen and the chick's body gained 35 mgm. so the loss by oxidation was 125 mgm. Evidently the original contention of Iljin, that the spare yolk is used much more for energy than for storage, received confirmation through the work of Romenski, and the efficiency would here be extremely low, about 20 per cent. Romenski's figures permit us to compute what intensity of protein combustion goes on under these conditions. The 125 mgm. of nitrogen

[1] Later work by Roberts; Parker; Holmes, Halpin & Beach, and Heywang & Jull, agrees with Schilling & Bleecker's view on this point.

disappearing may be regarded as wholly protein nitrogen, and will therefore correspond to 783 mgm. of protein. Now the maximum intensity of protein combustion within the egg is 80 mgm. per 100 gm. wet weight of embryo per day, and here we have 783 mgm. per 30 gm. wet weight of embryo per $1\frac{1}{2}$ days, or 1740 mgm. per 100 gm. per day. There seems little doubt but that in early post-natal life a utilisation of protein can go on which does not seem to be possible at any earlier stage although the protein is there. This fact fits in remarkably with a number of others and leads to certain speculations of much interest, which will be brought forward in Section 9·15.

Table 115.

| | Uric acid excretion per 100 gm. wet weight of body per day (mgm.) | Protein catabolism in % of the total material catabolised | Investigator |
|---|---|---|---|
| Adult mammalian liver (*in vitro*) | — | 4–27 | Singer & Pöppelbaum |
| Adult hen | | | |
| (*a*) Inanition after corn diet | 50–100 | 10–14 | Von Knierem ; Schimanski |
| (*b*) During corn diet ... | 50–100 | 6 | and Voltz |
| Newborn chick | | | |
| Inanition, except for yolk ... | 127 | 5–6 | Fridericia and Bohr & Hasselbalch |
| Chick embryo | | | |
| 14th to 17th day of incubation | — | 5–6 | Fridericia |
| Throughout incubation ... | — | 2–3 | ,, |

It also raises the question of what relation exists between the combustion of protein by the chick in the egg and by the adult hen. It occurred to Fridericia to make a comparison of this sort. Referring to the work of von Knierem and of Schimanski he calculated that the adult hen on an ordinary corn diet, or in a fasting period after such a diet, would excrete an average amount of 0·5 to 1·0 mgm. of uric acid per gm. body-weight per day. This would be 100 mgm. of uric acid per 100 gm. body-weight per day or 206 mgm. of protein catabolised per 100 gm. body-weight per day, i.e. more than twice as much as the highest point of pre-natal protein catabolism. Fridericia collected the excrement of newly hatched chicks for a day or two after birth, and did not find such large amounts of uric acid as would be expected from Romenski's figures. He found 53·2 mgm. of uric acid per kilo per hour, i.e. 127·5 mgm. uric acid per 100 mgm. per day corresponding to 266 mgm. of protein combusted per 100 gm.

per day, instead of 1740 mgm. However, this was a good deal more than the highest point reached in pre-natal life. Fridericia did not work out the percentage participation of protein in the total combusted material by actual measurements of the fat and carbohydrate disappearing, as was done in later work (cf. p. 1134) but knowing the total heat output from Bohr & Hasselbalch's figures, and knowing the amount of uric acid produced, and hence the amount of protein catabolism and its heat, he calculated the latter in per cent. of the former. Thus he was able to draw up the above table. It must be remembered that the above data for the chick embryo were Fridericia's own, and that other workers on uric acid formation in the egg do not wholly confirm them, but nevertheless, a comparison with these (Table 141) will show that the main conclusions to be drawn are not affected by these discrepancies. The most probable alteration which should be made in the table is a lowering of the last value. Further researches should be undertaken to decide the point at issue between Fridericia and Romenski.

## 6·12. Maternal Diet and Embryonic Constitution

On p. 248 I discussed the extent to which changes in the diet of the hen could influence the chemical constitution of the egg and thence perhaps of the embryo. In the case of the mammal it is obviously impossible to trace the effects of the diet upon the constitution of the egg, but something has been done on its effects on the constitution of the embryos at birth.

Reeb studied the effects of under-nutrition in rabbits upon their offspring and found that the experimental rabbits produced embryos 41·2 per cent. lighter than the controls, containing 44 per cent. less dry solid, and 61·9 per cent. less fat. The same results emerged, with only minor differences, from work with dogs. Paton's guinea-pigs gave the following results:

| | Weight of young per gm. of mother at term (gm.) | Average no. of young per litter |
|---|---|---|
| Well-fed ... ... | 0·350 | 2·7 |
| Under-fed ... ... | 0·248 | 2·5 |

Zuntz and Bondi worked with rats—the former confirmed Reeb, and the latter reported that a rich fat diet caused an unusually high fat content of the embryos. On cows, Eckles observed no effect of

moderate underfeeding. As for man, Prochovnik in 1889, on clinical grounds, affirmed that the infants of women on restricted diet were smaller and lighter than those of women on liberal diet. During the European War of 1914–1918, which unfortunately provided the German workers with many opportunities of studying this problem, Prochovnik's views were not substantiated. Peller, it is true, found a slight difference in weight, but Momm; Sörgel; Ruge and many others could not obtain any evidence for it. Two considerations, advanced by Zuntz, explain why these results differ from those of the animal researches—(1) in spite of the war diets, the deficiency was not enormously great, and (2) the birth-weight in man is a much less percentage of the maternal weight than in other mammals (see p. 475).

Dibbelt put pregnant dogs on a calcium-poor diet, and found that the calcium content of the embryos at term was normal. Zuntz's work on rats included an experiment of this kind, which gave equally negative results. But on the other hand Fetzer found that iron-rich diets increased the iron-content of the foetuses, while iron-poor ones, if below a certain level, led to abortion and loss of the litter. The effects of vitamine deficiency and excess on the embryos have also been studied, but for an account of the results obtained reference must be made to Section 16·5.

The strain on the maternal organism of providing material for foetal growth is strictly outside the scope of this book, but a word or two on the subject may be said here. Gowen investigated the extent to which the milk yield of cows is reduced by gestation, and after a close analysis of extensive statistics concluded that a cow in the 9 months of pregnancy produces 342 to 628 lb. of milk less than a non-pregnant one. There is thus a 5 per cent. diversion of milk products to the foetus, a diversion, moreover, which would seem to bear equally on all the constituents of the milk, for the butterfat percentage, for example, is not influenced at all. Very similar results were obtained by Brody, Ragsdale & Turner, who found a diversion to the foetus of 450 lb. of milk, i.e. rather more in dry weight than the foetus at birth which is equivalent to 275 lb. of milk. In mice, according to Kirkham, the increased gestation time of nursing animals seems to be due rather to abnormalities of implantation than to any drain on the maternal body.

For the considerable literature on the effect of alcohol and other

drugs on birth weight, reference may be made to the papers of Hanson & Heys.

In this connection it is interesting to find general agreement existing that the larger the litter in a mammal the smaller each individual is. Thus Bluhm found the following figures in mice:

| No. of embryos in litter | Weight in gm. of each individual |
|---|---|
| 2 | 1·38 |
| 4 | 1·26 |
| 6 | 1·19 |
| 8 | 1·12 |
| 10 | 1·07 |

And the same holds true for the guinea-pig (Ibsen & Ibsen), the rabbit (Kopeć) and the pig (Hammond).

## SECTION 7

# THE ENERGETICS AND ENERGY-SOURCES OF EMBRYONIC DEVELOPMENT[1]

### 7·1. The Energy Lost from the Egg During Development

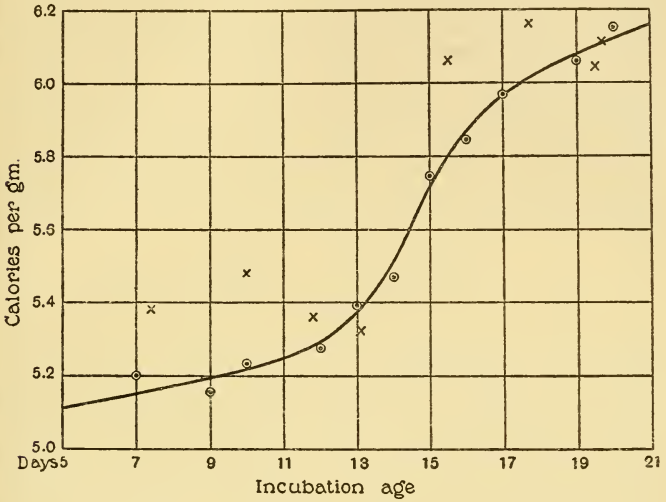
Many investigators, realising that, owing to the changing composition of the embryonic tissues and the raw material of development, it is difficult to compare different entities if the material exchange is alone considered, have thought it worth while to investigate the energetics of the transformations in question. Sometimes this has been done, as we have already seen, by measuring the heat produced during a given developmental period by the embryonic tissues, sometimes it has been done by combusting the embryo and the yolk in the bomb calorimeter, and obtaining in this way data for the amount of energy stored in the substance under investigation. It is with researches of the latter type that this chapter will largely be concerned.

To establish first the fact that the calorific value of an equal amount of a definite substance may change during the developmental period, it is simply necessary to refer to the figures of Murray, who in 1926 estimated the fuel value of 1 gram of dry substance in the chick embryo between the 5th and the 21st days of incubation. The curve he obtained is shown in Fig. 256, which clearly shows that it rises in a sigmoid form, the points agreeing well enough with the earlier values of Tangl. The increase in calorific value is no doubt due to the decrease of the inorganic and the increase of the organic quota in the embryo. From Fig. 256 it can be seen that the calorific value of 1 gm. of dry embryo at the end of incubation is about 6·2, having risen from 5·1 at the 5th day, and Murray pointed out that it was rising up towards the level of the calorific value of the unincubated yolk and white taken together, i.e. 6·94. The variation in fuel value here shown reveals well the drawbacks of the plastic efficiency coefficient. As a measure of efficiency it does not take account of the fact that the units on which it is based are constantly changing in calorific quality.

It is interesting that Murray found a divergence between the observed and calculated calorific value in the first half of incubation.

[1] Throughout this book "calorie" means gram-calorie and "Calorie" kilo-calorie.

This was rightly regarded by Murray as outside the limits of the standard errors. He concluded that either or both of the two constants used in the calculations were too high for the protein and fat of the embryo during the early stages of incubation, for, after all, these constants have been exclusively derived from experiments on adult tissues. It may, therefore, be assumed as probable that




⊙ Murray. × Tangl.

Fig. 256.

the true calorific constants for the substances present in early embryonic life (in the case of the chick) should be regarded as lower than those for the corresponding adult substances. It follows, then, that, just as the fuel value per gram of organic matter rises with age, so also the fuel value of either or both the protein and fat fractions rises, due to the increasing proportion within each group of substances with a relatively high calorific value.

The opening up of the study of energy-relations in embryogenesis is due to Tangl and his collaborators, who in a long series of papers from 1903 onwards published the results of their extensive researches with the bomb calorimeter. Eichwald's review presents some of their

data. Tangl's first paper dealt with the avian egg, and he defined his aim as the attempt to see how much energy was utilised during development, in what manner the process went on, and what were the sources of it. He was profoundly impressed by the difficulty and the fascination of the problem of "work of development", the problem of relating the morphological and structural coming-into-being with physico-chemical work done. It was not possible, he felt, that living animals should acquire ontogenetically their shape and form, without having to pay a fee, perhaps a heavy one, to entropy. Obviously this question is one of some difficulty, and a good deal depends on definitions, in which respect Tangl and his school were not altogether happy. "Die Menge der während der Entwicklung des Embryos umgewandten chemischen Energie, nenne ich Entwicklungsarbeit", said Tangl. Thus the first figures he obtained (on the egg of the starling)

| | cals. |
|---|---|
| Undeveloped egg ... | 3067 |
| Finished embryo ... | 2312 |
| Entwicklungsarbeit | 755 |

showed the method he intended to adopt. In calling the total amount of energy not used for storage in the embryo "Entwicklungsarbeit", Tangl was confusing two things which it is important to keep separate, (1) the amount of energy used by the formed cells of the embryo during development for combustion processes, and disappearing as heat, and (2) the amount of energy, if any, which passes into the embryo in the form of food from the yolk and white, and which is yet not recoverable from the dried material by the bomb calorimeter. This second fraction has more right to be called "Entwicklungsarbeit" than the first one, for as soon as any new cell is formed it begins an oxidative metabolism of its own. Undeniably this is work done during development, but not a true "Entwicklungsarbeit", the energy of which would have to be put down on the balance sheet of ingoings and outgoings as missing, i.e. in some way bound up with the structure. Exactly how this could take place has been the theme of several speculations; thus some have suggested that energy would be required to maintain certain orientations of molecules at intracellular surfaces, and doubtless the form of the animal is the outward and visible sign of such inward steady states, but these postulated processes have never been demonstrated. There is

also, of course, osmotic work and secretion work to be considered. I shall return later to this point; meanwhile, it is necessary to penetrate further into the facts concerning "Entwicklungsarbeit". The terminology presents difficulties, but the method adopted will be to speak of the "Entwicklungsarbeit" in Tangl's sense as Ea. and of the true "Entwicklungsarbeit", if there is any such thing, as O.E. or organisation energy. Thus Ea. will be defined as the amount of energy not stored in the embryonic tissues and not left behind in the unused raw materials at the end of development, while O.E. will be defined as the amount of energy, if any, laid up in the embryo, which, though appearing as calorific value of combusted wet tissues, would not result from the combustion of an unorganised mixture of its constituents[1]. If all the constituents of the finished embryo could be mixed together mechanically and the mixture then combusted, the calories contained in it might be slightly fewer than those contained in the same substances when organised into an embryo. This difference is the O.E. These conceptions are illustrated by the diagram in Fig. 259, to which reference should be made.

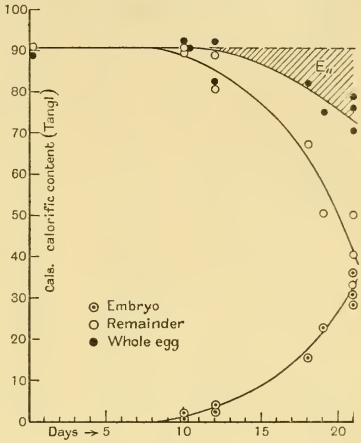



Fig. 257.

Tangl's first experiments, then, showed that the Ea. of the starling's egg was 755 cal., i.e. 24·6 per cent. of the original amount of energy present, corresponding to a loss of dry weight during development of 15·7 per cent.

His next experiments were done on hen's eggs of three races, all of which had dry weights when unincubated varying between 24·3 and 24·9 per cent., and calorific values of between 6906 and 7078 cal. per gram. dry substance, and between 1692 and 1734 cal. per gram wet substance. The whole egg contained 88·9 Cal. What

[1] Definitions of this and other terms will be found summarised together on p. 999.

happened on incubation is shown in Fig. 257. The energy contained in the tissues of the embryo increased steadily with its growth; that contained in the remainder of the egg equally steadily decreased, but in addition there was, of course, a loss from the egg as a whole due to the combustions. This loss appears on the graph as a shaded area, the extent of which at the end of development represents the Ea. and amounts to 16 Cal. This is more energy than is spent by the starling embryo during its development, but the chick embryo is larger than that of the starling. In order to have a common basis of comparison, Tangl computed the Ea. as related to 1 gm. of embryo (wet weight), which he called the "relative Entwicklungsarbeit" (hereafter referred to as the R.Ea.), and as related to 1 gm. of embryo (dry weight), which he called the "specifisches Entwicklungsarbeit" (hereafter referred to as the S.Ea.). When these values were calculated out for the chick, he obtained the graph shown in Fig. 258. The Ea., of course, rose during development, for the quantity of material burned on any one day rose, but the R.Ea. and the S.Ea. fell, as they were bound to do, owing to the declining metabolic rate. Tangl introduced some corrections at this stage for the weight of the membranes in the early stages, but, even after these had been made, the R.Ea. and the S.Ea. were still much higher for the earlier than for the later stages. In other words, more energy was dissipated in forming 1 gm. of 10th day embryo (wet or dry weight) than in forming 1 gm. of 21st day embryo, a conclusion quite in harmony with all that is known about the change in respiratory intensity with age.

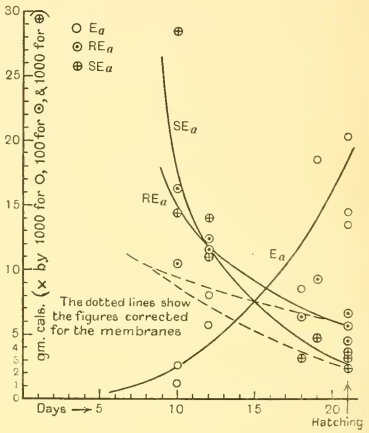



Fig. 258.

Tangl compared the magnitude of R.Ea. in the chick embryo with the "Erhaltungsarbeit" or basal metabolism of the adult hen; an

average figure for the chick was 100 cal. and for the adult hen 71, other conditions being maintained as equivalent as possible. He concluded, also, that the Ea. was derived almost entirely from fat, by dividing the amount of dry solid disappearing from the egg by the amount of chemical energy disappearing, thus finding that the calorific value of the material disappearing was, on an average, 9000 cal. per gm. He then combusted some purified egg-fat and obtained a calorific value of 9476 cal. per gm. Tangl did not fail to point out how well this fitted in with the results of the chemical analyses of Prévost & Morin; Liebermann, and Pott.

Table 116.

| Day | Cals. Stored | Cals. Combusted (Ea.) | Total | Efficiency of storage. Storage × 100/storage and combustion |
|---|---|---|---|---|
| 10 | 0·269 | 1·42 | 1·689 | 15·9 |
| 10 | 1·04 | 2·76 | 3·8 | 27·4 |
| 12 | 2·2 | 5·81 | 8·01 | 27·5 |
| 12 | 3·78 | 8·08 | 11·86 | 31·9 |
| 18 | 15·58 | 8·35 | 23·93 | 65·0 |
| 19 | 23·89 | 18·79 | 42·68 | 56·0 |
| 21 | 27·34 | 13·51 | 40·85 | 65·0 |
| 21 | 36·68 | 20·09 | 56·77 | 65·0 |
| 21 | 31·63 | 14·30 | 45·93 | 69·0 |

Tangl next considered the relations between Ea. and the energy stored up in the tissues of the embryo, though he did not discuss this from the point of view of efficiency. Nevertheless, the table he drew up is interesting, and is reproduced in Table 116. As development goes on, the relation between storage and combustion changes, for while at first the efficiency of storage is low, it rises as development proceeds, and reaches 66 per cent. or so by the end of incubation. This course of events has since been repeatedly confirmed. Tangl expressed it by concentrating attention on the fact that the body of the finished chick embryo contains roughly 32 Cal. of chemical energy, and the Ea. is roughly 16 Cal., therefore about $\frac{2}{3}$ of the original energy was used for storage, and $\frac{1}{3}$ for combustion. The overall energetic efficiency was therefore 66 per cent. Tangl's figures for calorific value of the embryonic tissues have already been discussed in relation to the similar ones obtained by Murray—it is worth noting, however, that Tangl did not find any notable alteration in the calorific value of the unused yolk between the 10th and 21st

days of incubation. The distribution of chemical energy in the finished embryo was as follows:

Table 117.

| Organ | Dry weight in grams | calories | % of the energy-content of the whole embryo | calories per gram dry weight |
|---|---|---|---|---|
| Muscles ... ... ... | 1·3391 | 8,951 | 28·3 | 6687 |
| Central nervous system ... | 0·1642 | 986 | 3·1 | 6007 |
| Viscera ... ... ... | 0·9329 | 5,551 | 17·6 | 5950 |
| Skin, etc. ... ... ... | 1·1927 | 6,756 | 21·4 | 5537 |
| Bones ... ... ... | 1·4461 | 7,094 | 22·4 | 4907 |
| Remainder ... ... ... | 0·4502 | 2,288 | 7·2 | 5647 |
| Whole embryo ... ... | 5·4801 | 31,626 | 100·0 | 5771 |
| Membranes ... ... | 0·2818 | 1,220 | — | 4329 |

To a large extent these figures reflect the varying fat-content of the individual parts of the body. Comparative researches on this subject at different stages of development might reveal some interesting relationships.

The paper of Tangl & v. Mituch contained a more accurate investigation of the energy relations in the hen's egg. The individual differences between energy-content of embryos from different hens, etc., were found to be exceedingly small; and the average figure for the Ea. was 22·94 Cal. This was distinctly higher than the corresponding value given by Tangl in his first paper, and it meant, of course, that the R.Ea. and S.Ea. were also higher than he had at first thought. The following table shows the figures for six embryos:

Table 118.

| | calories | | | |
|---|---|---|---|---|
| | Ea. | R.Ea. | S.Ea. | Specific energy-content of the substance burnt |
| From hen (*a*) | 20,460 | 727 | 3830 | 9,250 |
| | 22,940 | 821 | 3640 | 10,510 |
| | 23,130 | 993 | 4860 | 9,070 |
| From hen (*b*) | 23,640 | 709 | 3260 | 10,460 |
| | 24,200 | 826 | 3810 | 9,840 |
| | 23,240 | 743 | 3400 | 9,560 |

so that the average result was R.Ea. 805 cal. and S.Ea. 3600 cal.

Tangl's second paper was concerned with an attempt to carry out his ideas, working with various bacilli during the development of cultures. His work in this field will be found assessed in Stephenson's

monograph. The third paper of the series was by Farkas, and contained a careful study of the developing silkworm egg from the energetical point of view. He made complete analyses of the eggs before and after their development, the details of which receive consideration elsewhere in this book. For the unincubated egg he got the following values:

| | |
|---|---|
| calories per gram ... ... | 2163 |
| calories per gram dry weight... | 6104 (specific energy-content) |
| calories per gram fat ... ... | 9343 |

for the hatched larvae:

| | |
|---|---|
| calories per gram ... ... | 1631 |
| calories per gram dry weight... | 5782 |

and for the unused materials, membranes, etc.:

| | |
|---|---|
| calories per gram ... ... | 4560 |
| calories per gram dry weight... | 5301 |

Or, in round numbers, expressed differently, i.e. for the whole material:

| | Calories in the material used | % of the value for the unincubated eggs |
|---|---|---|
| Unincubated eggs ... ... ... | 71·402 | — |
| Hatched larvae ... ... ... | 31·879 | 44·65 |
| Unused material, membranes, etc. ... | 22·291 | 31·22 |
| Material lost, i.e. used up during development ... ... ... ... | 17·232 | 24·13 |

The analytical figures showed that 17·32 per cent. of the original dry solid, 48·24 per cent. of the original fat-content, and 0·65 per cent. of the original nitrogen-content had disappeared, so at first sight it seemed certain that the source of the energy utilised had been fat. However, as the nitrogenous end products were not estimated, and as they would remain in the eggs and so form part of the nitrogen value at the end of development, some protein may have been burned too, and perhaps some of the fat was turned into carbohydrate, of which no determinations were made.

From the figures just given, it follows, as 42,220 eggs were used, that in the development of one silkworm egg 0·408 cal. is required for waste or combustion, i.e. the Ea. is equivalent to 0·408 cal. or 0·174 mkg. From the same figures the R.Ea. may easily be calculated, and Farkas found that it came to 882 cal., while the S.Ea. was

3125 cal. Farkas was naturally struck with the resemblance between these figures and those which in the previous summer had been found to hold for the hen's egg by Tangl, i.e. R.Ea. 658 cal. and S.Ea. 3426 cal. Considering that the hen's egg weighs 70,000 times as much as the silkworm's egg, and the hatched chick 50,000 times as much as the hatched silkworm, the agreement was remarkable. It meant that, in order to produce 1 gram of finished embryo, whether of the silkworm or the chick, and whether wet or dry weight was considered, about the same amount of energy was required for combustion purposes. It meant that in each case about the same degree of wastefulness was found in embryonic development; thus the average overall number of calories per gram of finished chick (dry weight) was 5771, and the average number of calories wasted in producing this result was 3426; therefore the work was done with an efficiency of 62·9 per cent. $\left(\frac{5771}{5771 + 3426} \times 100\right)$. Tangl and his associates, however, did not emphasise this aspect of the question, for they were more interested in the problem of the relations between energy and form. They did not look on the energy of the substances combusted as energy wasted, i.e. as energy lost *during* development, but rather as energy associated in some way with the assumption of structure and form, i.e. as energy lost *for* development.

Table 119.

| | Used during development | | | | | |
|---|---|---|---|---|---|---|
| | Dry solids | | Fat | | Other solids | |
| | Weight (mgm.) | Energy (cal.) | Weight (mgm.) | Energy (cal.) | Weight (mgm.) | Energy (cal.) |
| 1 gm. larva (R.Ea.) | 103·6 | 882·0 | 59·9 | 559·0 | 43·7 | 323·0 |
| 1 gm. dry weight larva (S.Ea.) | 367·8 | 3125·0 | 212·1 | 1982·0 | 155·2 | 1143·0 |
| 1 larva (Ea.) | 0·048 | 0·41 | 0·028 | 0·26 | 0·02 | 0·15 |

Farkas went on to point out that, during the development of 19·54 gm. of silkworm larvae, 2·03 gm. of dry substance had disappeared from the eggs, corresponding to 17·23 Cal. of energy. The specific energy-content of the substance lost (energy per gram dry weight) was 8·51 Cal., which differed from the results obtained by Tangl on the hen, i.e. between 9 and 10 Cal. The analytical figures permitted Farkas to conclude that the Ea. of the silkworm's

egg was provided to the extent of 63·4 per cent. by fat combustion and 36·6 per cent. by the combustion of some other substance or substances, of which protein was probably the most important, though, from Tichomirov's earlier work, some carbohydrate was probably also utilised. Table 119 illustrates these facts. By running one series of larvae right through after hatching during a hunger period of some days, Farkas was able to get some idea of the energy relationships during this post-hatching period, during which the remains of the yolk are used. As Table 120 shows,

Table 120.

| | | Loss of matter and energy | | |
|---|---|---|---|---|
| | | Embryonic period + hunger period | Embryonic period only | Hunger period only |
| Dry weight | Gm. | 0·1786 | 0·1036 | 0·0750 |
| | % of undeveloped | 30·4 | 17·32 | 13·08 |
| Fat | Gm. | 0·1060 | 0·0599 | 0·0461 |
| | % of undeveloped | 79·77 | 48·24 | 31·53 |
| Energy | Cal. | 1·410 | 0·882 | 0·528 |
| | % of undeveloped | 40·23 | 24·13 | 16·10 |

the values for the post-embryonic period are all lower than those for the time before hatching. Comparison of these results with the analytical figures indicated that substances of lower energy-content than either fat or protein were combusted for energy during this period.

Tangl & Farkas next published a joint paper on the development of the trout embryo. They found that 1 egg (presumably of *Salmo fario*) weighed 88·2 mgm., and contained 193 cal. energy. The specific energy-content (i.e. per 1 gm. dry weight) was 6453 cal. For 518 eggs the energy-content before development was 99·85 Cal., and after it 96·39 Cal., showing a loss of 3·46 Cal. during the process, or for one egg 6·68 cal. Neither nitrogen nor fat diminished—in fact, the latter rose by 37 per cent.—a circumstance which led Tangl & Farkas, after various experiments, to the suggestion that urea and uric acid were acting as sources of energy. This remarkable assumption has since turned out to be unnecessary, and will be discussed later (see p. 1118). Tangl & Farkas could not calculate the R.Ea. and the S.Ea. for they were unable to ascertain the weights of the embryos at the various stages.

Tangl & Farkas made a comparison between the three kinds of eggs they had studied, as follows:

| | Loss in % of the original amounts from fertilisation to hatching | | |
|---|---|---|---|
| | Trout | Hen | Silkworm |
| Total weight ... ... | 5·6 | 17 | 26 |
| Water ... ... ... | 7·1 | 21 | 69 |
| Dry solid ... ... | 2·7 | 18 | 17 |
| | Loss in % of the total loss | | |
| Water ... ... ... | 84 | 85 | 77 |
| Dry solid ... ... | 16 | 15 | 23 |

The slight loss of water from the trout's egg shown by Tangl & Farkas does not contradict the findings of Kronfeld & Scheminzki, for, as Fig. 238 shows, before hatching the water-content of the larva as a whole is almost constant, although that of the yolk alone is decreasing. The Ea. of 6·68 cal. was a remarkably small proportion of the energy originally contained in the egg, only 3·5 per cent., and contrasted with the 18 per cent. which is lost by the chick embryo by the time of hatching, and the corresponding 24 per cent. of the silkworm. But it must be remembered that hatching occurs relatively early in the trout, and that for a long time afterwards the yolk is the only source of food for the larva. The R.Ea., then, as Tangl & Farkas pointed out, would have been distinctly lower than that for the other embryos. Here we touch one of the fundamental difficulties of Tangl's conceptions, for, when we define the S.Ea. as the amount of energy used for combustion during the storage of, i.e. the formation of, 1 gm. dry weight of the finished embryo, we omit to define what a finished embryo is. Tangl assumed throughout his work that the time of hatching was the natural index, but in the case of organisms such as the trout, which have a prolonged yolk-sac period, this is evidently wrong. It is probable that, if one were to take the yolk-sac period into consideration, one would find an R.Ea. very like those for the chick and the silkworm, but this has not so far been done.

## 7·2. Energy of Growth and Energy of Differentiation

The sixth and seventh papers of the series were devoted by Tangl to a study of the energetics of metamorphosis in the fly, *Ophyra cadaverina*, and to a general discussion of the meaning of "Entwicklungsarbeit" in relation to embryonic growth and insect metamor-

phosis. Here he distinguished between "Erhaltungsarbeit" or basal catabolism, i.e. energy of maintenance, on the one hand, and "Arbeit für Bildung von lebenden Substanz", on the other hand, but he regarded the former as negligibly small during embryonic development, probably a rather important error. The "Bildungsarbeit" he divided into "Neubildungsarbeit" and "Wachstumsarbeit". Roughly corresponding to differentiation and growth respectively, these terms stood for the production of organs and the laying down of morphological and chemical structures in an architectural plan, on the one hand, and the actual increase in size of individual cells and the body as a whole, on the other hand. Tangl hit upon an ingenious method which he hoped would solve the problem of assessing how much of the "Entwicklungsarbeit" was devoted to "Neubildungsarbeit", and how much to "Wachstumsarbeit", i.e. the study of insect metamorphosis. There, Tangl argued, the weight loss was very slight, practically negligible, no food was taken in, there would be no "Wachstumsarbeit" (Wa.), and all the changes could be regarded as the rearrangement of a pre-existent pattern, alterations of the form and spatial arrangement of the cells making up the various organs. Everything would be "Neubildungsarbeit" (Na.). This proposal, interesting as it was, was from the first open to criticism. It was well known that the pupa has a definite, if feeble, respiration, and therefore could hardly be considered as losing no weight. Moreover, Tangl's calculation depended upon the assumption not only that

$$\text{Ea.} = \text{Wa.} + \text{Na.},$$

which is probably not true, but also upon the assumption that the "Umbildungsarbeit" (Ua., i.e. transformation work) of metamorphosis was equivalent to the Na. In other words, practically no attention was paid by Tangl to the histolysis of the old arrangements, although he was expressly studying a holometabolic insect. Why should there not be a "Histolysearbeit" (Ha.)? It is well known to builders and contractors that "Histolysearbeit" may be considerable. If there were, Ua. would be equivalent to Ha. + Na., and, as no method was devised for distinguishing between these two, the subtraction of Na. from Ea. to get Wa. was invalid. Again, the insect larva and pupa contains a "fat body" which, according to Folsom and many entomologists, can be considered as the equivalent

of a yolk, and which disappears in metamorphosis. Tangl was here making the same mistake as has so often been made by other investigators of closed systems such as eggs. A given substance increases in amount, therefore, they say, it cannot be in process of being used up, forgetting that there may be a balance between catabolic and anabolic factors, the net result of which happens to be in favour of the latter. So here, the "fat body" may be responsible for a good deal of Wa. in metamorphosis.

Tangl used for his work a large supply of the larvae of *Ophyra cadaverina*, one of the corpse flies, and carried out on it a large number of chemical analyses and bomb calorimeter measurements. Thus, during the 6 days of pupation, 1000 pupae combusted 3·72 Cal. of energy, or per day 0·62 Cal., and during the $13\frac{1}{2}$ days of metamorphosis the pupae combusted 3·82 Cal., or 0·282 Cal. per day. Thus the energy utilisation per gm. per day during the first period was 57·2 cal., and during the second period 36·0 cal. From his chemical analyses Tangl calculated that, in the pupation period, 88·7 per cent. of the energy in the solid burned was provided by fat, and that, in the metamorphosis period, 98·6 per cent. was so provided.

From the figures given, it follows that during the metamorphosis of the completely pupated larva into the completely free imago 3·82 cal. are lost per insect. To this must be added the calorific value of the excrements in the chrysalis and the chrysalis case itself, i.e. 4·44 cal., making a total of 8·26 cal. As the imago when completed weighed 7·32 mgm. (all these values, of course, were averages from a large number of observations) the R.Ea. and the S.Ea. were readily calculable, and came out as follows:

| | calories |
|---|---|
| R.Ea. ... ... | 523 |
| S.Ea. ... ... | 1566 |

though these should perhaps be termed R.Ua. and S.Ua. With these figures Tangl compared some other values which he calculated from the chemical work of Weinland on the fly *Calliphora vomitoria* thus:

| | calories |
|---|---|
| Ea. ... ... | 24·3 |
| R.Ea. ... ... | 399 |
| S.Ea. ... ... | 1184 |

The agreement was striking, but would have been even more so had Weinland counted the abandoned chrysalis cases as part of the waste,

instead of part of the fly; when Tangl's figures were computed in that way, they came to R.Ea. 462 cal. and S.Ea. 1144 cal., almost in exact correspondence. Tangl next compared these results with those of Farkas on the silkworm's metamorphosis, and found that, though the Ea. of the silkworm was much higher than that of either *Ophyra* or *Calliphora* (it is a much bigger insect), its R.Ea. and S.Ea. were very similar:

| | calories |
|---|---|
| Ea. ... ... | 379 |
| R.Ea. ... ... | 481 |
| S.Ea. ... ... | 1962 |

from which he concluded that the energy wasted by combustion in the production of 1 gm. weight of imago wet or dry from the larval stage was much the same for the two diptera and the lepidopteron.

The next step in Tangl's calculations was to find out how much energy had to be given off in order to transmute the larva into the pupa. Knowing the weights of the organism at the beginning and end of the pupation period, and having the results of bomb calorimeter measurements at hand, these values were obtained:

| | calories | |
|---|---|---|
| | *Ophyra cadaverina* (Tangl) | *Bombyx mori* (Farkas) |
| Pupation period (larva to pupa) | | |
| Ea. ... ... ... ... | 4·34 | 416 |
| R.Ea. ... ... ... ... | 467 | 317 |
| S.Ea. ... ... ... ... | 1157 | 1429 |
| Metamorphosis period (pupa to imago) | | |
| Ea. ... ... ... ... | 3·82 | 379 |
| R.Ea. ... ... ... ... | 523 | 481 |
| S.Ea. ... ... ... ... | 1566 | 1962 |

By adding the results together so that the energy-consumption for the whole period, i.e. from the beginning of pupation to the birth of the adult imago, Tangl got the following figures:

| | *Ophyra cadaverina* | *Bombyx mori* |
|---|---|---|
| Ea. ... ... ... | 8·16 | 795 |
| R.Ea. ... ... ... | 1115 | 1032 |
| S.Ea. ... ... ... | 3344 | 4115 |

which, as he did not fail to notice, are of the same order as those for embryonic development.

This relation he elaborated at length in the seventh paper of the series. As can be seen from Table 121, where all the relevant data are collected, it was very definitely the case that the period of true

metamorphosis, i.e. from complete pupation to free imago, had an R.Ea. and an S.Ea. of about half the value for pupation plus metamorphosis, and, more significantly, half that for embryonic development. As, in Tangl's belief, metamorphosis consisted only of "Neubildungsarbeit" with very little "Wachstumsarbeit", the conclusion naturally followed that the partition between the two was probably 50 per cent. Inspection of Table 121 shows clearly that, for the formation of a gram of dry weight of finished embryo, an approximately equal amount of energy has to be used up, and this irrespective of the position of the animal in the taxonomic scale. Tangl's remark that "die spezifische Entwicklungsarbeit der tierischen Organismen keine Funktion ihrer phylogenetischen Stellung und Organisationstufe ist" may be said to be true, though, in spite of the remarkable correspondence between the silkworm and the chick, it would be desirable to extend the number of well-authenticated cases. Tangl also laid much emphasis on the fact that the energy utilisation was much faster in embryonic development than in metamorphosis, in the case of the silkworm, for instance, being as 2·97 : 0·61, or per day per gram 198 cal. in embryonic life and 33 in metamorphosis. "Die embryonale Entwicklung", said Tangl, "beansprucht also einen viel grösseren und intensiveren Umsatz von chemischer Energie als die Metamorphose; sie erfordert eine grössere und intensivere Arbeit." Tangl admitted that an unknown proportion of his "Entwicklungsarbeit" in embryonic development and his "Umbildungsarbeit" in metamorphosis was really "Erhaltungsarbeit", "energie d'entretien", or maintenance catabolism, but he thought it possible that this fraction was identical in the two processes. Thus the way was laid open for the subtraction of the metamorphosis S.Ea. from the embryonic S.Ea. He himself never actually made this calculation, but it was obvious from his figures that the average values for metamorphosis were R.Ea. 437 and S.Ea. 1550 cal., while those for complete development, when halved, were R.Ea. 422 and S.Ea. 1270 cal. The energy of development, then, would be regarded as being approximately equally divided between differentiation and growth-processes. Tangl did not, however, by any means commit himself to this conclusion, for he also suggested that the main component of the energy burned during metamorphosis was "Erhaltungsarbeit". At this point, indeed, Tangl and his associates came up against the difficulty which so often confronts those who try to relate chemical

## Table 121.

| Organism and period | calories | | | Duration of the process in days | Wet weight of the product at the end | calories in 1 gm. wet weight of finished embryo | calories in substance burned per day by the organisms | | calories in 1 gm. dry weight of finished embryo | Efficiency: $\frac{\text{Storage}}{\text{Storage} + \text{Combustion}} \times 100$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ea. | R.Ea. | S.Ea. | | | | per gram wet | per gram dry | | |
| *Embryonic development* | | | | | | | | | | |
| Starling (Tangl) ... ... ... | 755 | — | — | — | — | — | — | — | — | — |
| Hen, *Gallus domesticus* (Tangl) ... | 22000 | 810 | 3800 | 21 | 28·8 gm. | 1264 | 129 | 691* | 5771 | 62·9 |
| ,, ,, ... ... | — | — | — | — | — | — | — | — | 6148 | 67·0 |
| Silkworm, *Bombyx mori* (Farkas) ... | 0·408 | 880 | 3100 | 15 | 0·463 mg. | 1632 | 198 | 702 | 5325 | 63·2 |
| Minnow, *Fundulus heteroclitus* (Glaser) | 7·23 | — | 3280 | — | — | — | — | — | 4800 | 52·8 |
| Trout, *Salmo fario* (Tangl & Farkas) | 6·68 | — | — | 45 | 0·1 gm. | — | — | — | — | — |
| Polychaete Worm, *Sabellaria alveolata* (Fauré-Fremiet) | — | 34 | 111 | — | — | — | — | — | 5169 | 97·9 |
| Roundworm, *Ascaris megalocephala* (Fauré-Fremiet) | — | — | 280 | — | — | — | — | — | 5400 | 95·1 |
| Frog, *Rana temporaria*† (Fauré-Fremiet & Dragoiu) | 5·26 | 278 | 5260 | 20 | 17·8 gm. | — | — | — | 5500 | 51·1 |
| Toad, *Bufo vulgaris*‡ (Gayda) ... | 0·12 | 30 | — | 9 | — | — | — | — | — | — |
| *Metamorphosis* | | | | | | | | | | |
| Corpse-fly, *Ophyra cadaverina* (Tangl) | | | | | | | | | | |
| Larva to pupa ... ... ... | 4·34 | 467 | 1157 | 7 | 9·3 mg. | — | 44·1 | 193 | — | — |
| Pupa to imago ... ... ... | 3·82 | 523 | 1566 | 13·5 | 7·32 mg. | — | 26·5 | 115 | — | — |
| Larva to imago ... ... ... | 8·16 | 1115 | 3344 | 20·5 | 7·32 mg. | — | 32·6 | 146 | — | — |
| Blow-fly, *Calliphora vomitoria* (Weinland) | | | | | | | | | | |
| Pupa to imago ... ... | 24·3 | 399 | 1184 | 13·5 | 60 mg. | — | 26·6 | — | — | — |
| Silkworm, *Bombyx mori* (Farkas) | | | | | | | | | | |
| Larva to pupa ... ... ... | 416 | 320 | 1400 | 5 | 1·314 gm. | — | 57·2 | 178 | — | — |
| Pupa to imago ... ... ... | 379 | 481 | 1962 | 13·6 | 0·788 gm. | — | 36·0 | 107 | — | — |
| Larva to imago ... ... ... | 795 | 1032 | 4100 | 18·6 | 0·788 gm. | — | 43·9 | 133 | — | — |
| Hawk-moth, *Deilephila euphorbiae* (Heller) | | | | | | | | | | |
| Larva to pupa ... ... ... | 863 | 330 | 1322 | — | — | — | — | — | — | — |
| Pupa to imago ... ... ... | 970 | 373 | 1490 | — | — | — | — | — | — | — |
| Larva to imago ... ... ... | 1833 | 830 | 3320 | — | — | — | — | — | — | — |

* Murray's figure. † To the end of the yolk-sac period. ‡ To hatching.

with morphological events, namely, the difficulty of classifying morphological events in a really satisfactory way. In spite of all that had been done on metamorphosis by histologists, zoologists and naturalists, Tangl could not with certainty decide in what proportion growth and differentiation were proceeding; terms vague enough at best, and presenting almost equal difficulties in embryonic life. We here come face to face once more with that great impediment to research in these domains, the fact that we have no quantitative measure of differentiation (on this see Section 3·2 and the Epilegomena).

Before discussing the general outcome of Tangl's work, the eighth paper of his series must be mentioned. In it Glaser reported his estimations of the calorific value of the egg of a teleost, the minnow *Fundulus heteroclitus*. The figures came out as follows:

| | calories |
|---|---|
| 1000 eggs ... ... ... ... | 3273 |
| 1000 embryos (+membranes) ... | 2550 |
| | 723 |

As 1000 finished embryos weighed 0·535 gm. dry, the S.Ea. was 1350 cal. Glaser, however, realising that slightly more than half the weight of the embryo at hatching was unused yolk, doubled this figure, obtained an estimate of 3280, which was in good agreement with the rest of the figures obtained by Tangl and his school. Glaser also calculated that the specific energy-content of the substance burnt was 9·0 Cal., from which he concluded that the greater part of it was fat.

## 7·3. The Relation between Energy Lost and Energy Stored

Subsequent work by various authors brought forward figures which are shown in Table 121, but which do not agree with those of Tangl and his associates. This is probably due to the less accurate character of the later work. For the frog the figures of Fauré-Fremiet & Dragoiu, as can be seen from the table, differ somewhat from Tangl's, especially as regards R.Ea. and S.Ea., although the efficiency as calculated from them agrees well enough with the earlier work on the silkworm and the chick. This cannot be said of Fauré-Fremiet's experiments on the eggs of *Sabellaria* and *Ascaris*. Perhaps the divergence here is partly due to the difficulty in deciding just when embryonic development is complete, and the impossibility of separating the embryo from the yolk. Fauré-Fremiet's high levels of efficiency are probably illusory.

We may now return to the distinction made above, namely, that while no one could have taken exception to Tangl's ideas on "Entwicklungsarbeit" if it had been defined as the amount of energy disappearing in the solids combusted *during* a given amount of embryonic architectural work, yet throughout Tangl's writings the impression conveyed was that the "Entwicklungsarbeit" was the amount of energy disappearing *for* a given amount of architectural work. It is, of course, true to say that no embryonic growth, or any other kind of vital process, can go on without a certain wastage, for living machines are far from having an efficiency of 100 per cent., but there is no reason for supposing that the energy lost by combustion in the growing embryo is in any way quantitatively related to the actual increase of differentiated structures. It would, in fact, have saved a great deal of controversy if Tangl had expressed his results in terms of efficiency, for that is their real significance. To say that it involves a loss of 3100 cal. to build 1 gm. dry weight of silkworm, and that it involves a loss of 3280 cal. to build 1 gm. dry weight of minnow is simply to say that the work of storage which the fertilised egg-cell has before it cannot be accomplished without a certain amount of waste. In the case of the hen, the efficiency of storage is 62·9 per cent., in the case of the silkworm it is 63·2 per cent., in the case of the minnow it is 52·8 per cent., but this last value is certainly too small. Roughly it can be said that the efficiency of energy storage is in the neighbourhood of 66 per cent. in most of the cases known. This is so because the average calorific value of formed living tissue (average for whole body) is much the same. The important fact about Table 121, then, is not that the absolute values for Ea. come out so much alike, because, after all, the absolute calorific values for the tissues are alike, but that the relation between these is constant, and, in fact, that embryonic development goes on, as far as we can tell, with a constant efficiency in different animals.

But because Tangl apparently did not appreciate the real significance of his figures he was misunderstood from the first. Hammarsten, in an edition of his text-book which appeared during the publication of Tangl's series, showed that he did not understand Tangl's point of view. Then Bohr & Hasselbalch, in their paper on the heat production of the hen's egg, took over Tangl's expression "Entwicklungsarbeit", but used it in a quite different sense, namely, that of energy retained for organisation, or O.E. Bohr & Hasselbalch

felt that to speak of all the energy lost by the egg during its development as "work of development" was obviously wrong, for to do so is to assume that all the energy lost has been used for development proper apart from the maintenance of life. The only real sense, argued Bohr & Hasselbalch, in which the term "Entwicklungsarbeit" can be used, is to denote that portion of energy, if any, retained by the organism in the passage of fuel material into end-products. Krogh later supported this view. If there was any "cost of production" of embryo from yolk and albumen, then direct and indirect calorimetry would be expected to give different results. These considerations were among those which led Bohr & Hasselbalch to determine the oxygen taken in by the egg and the carbon dioxide and heat given out. As we have already seen (see p. 704 and Fig. 145), the curves for observed and calculated heat production were practically superimposable between the 8th and the 19th days of incubation. There was no retention of heat by the embryo, and therefore no true Ea., i.e. no O.E. But this statement has to be qualified by the proviso that their figures showed a discrepancy of 4 per cent., which might or might not have been heat retained.

These relationships are shown in Fig. 259. The original 87 Cal. of chemical energy present in the hen's egg at the beginning of incubation divides itself into 26 Cal. of unused yolk on the 21st day, 37 Cal. of embryonic tissues and 23 Cal. given off as heat from combustion. These form 30·4 per cent., 43·2 per cent., and 26·4 per cent. respectively of the initial provision. Side by side with the column showing the amount of energy which is contained in one hatching chick, another column is placed showing the amount of energy which would be present in a bottle containing all the compounds present in the chick, in their precisely correct concentration, but in the state of powders or liquids, i.e. entirely unorganised. It is evident that, as we have not a complete analytical balance sheet of all the substances present in the embryo, still less of their calorific values, intramolecular constitution, degree of activation, etc., we cannot at present measure the difference between these two columns, especially as there is reason to believe that it would be very small. However, a portion is marked off at the top of the right-hand column, and labelled O.E. It is to be supposed that Bohr & Hasselbalch's 4 per cent., if it is not simply due to errors of technique, would take its place as part of the O.E. It will be evident that Tangl's work tells us nothing at all about the O.E., or, as we defined it before, the

energy retained in a given amount of spatial intracellular and extracellular organisation. It is difficult to form a clear picture of this fraction of the energy. It is to be distinguished from Lapicque's

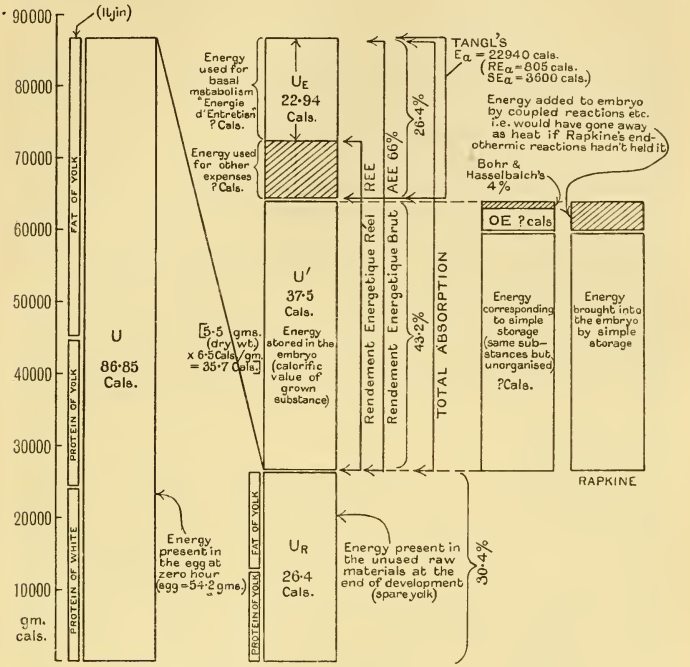



Fig. 259. This diagram represents the changes between 0 and 20 days' incubation. For chicks allowed to hatch naturally U′ will be larger; thus chick weight in % of egg-weight is 68 (Jull & Heywang; Upp), 66 (Jull & Quinn) or 64 (Halbersleben & Mussehl) according to the breed (see p. 249).

"epictesis", which is rather the work done by a secretion process, and it more resembles Shearer's conception of the work done in keeping the parts of cells and tissues together as physical systems. If this expense may be said to be in adult life of a definite though small magnitude, then, evidently, as the structure and organisation grows in embryonic life, this quota must also grow. In other words, the more organisation you have, the more molecules you maintain oriented a little differently from the position they would otherwise

adopt, the more the O.E. will be. It has often been maintained, e.g. by Johansson, that animals have no expenses of this kind to meet, on the ground that, when Meyerhof caused erythrocytes to cytolyse inside a calorimeter, he observed no increased heat production, yet the same worker's results on sea-urchin's eggs could be adduced on the contrary side (and see also p. 985). In any case, the complex processes of cytolysis would have to be eliminated in some way if a serious attempt was being made to assess the O.E. directly. The conclusion to which we come, then, is that one calorie of energy contained in yolk and white can transform itself into one calorie of energy contained in feathers, muscles, blood and brain, without any loss of energy except that necessitated by the living cells in their quality of living cells, i.e. more or less inefficient machines. But apart from this necessary expenditure of energy, apart from the universal income tax extorted from all living cells by virtue of their constitution, the transformations of the egg seem to go on without appreciable cost, and the organisation of the animal appears from nowhere, strangely devoid of physico-chemical antecedents. Such a conclusion is intellectually unsatisfactory, and in the future attempts will certainly be made to demonstrate the existence of a definite O.E. and to measure its magnitude[1].

It is necessary at this point to consider again the theoretical work of Rubner on the energy relations in embryonic life. His experiments on the storage of food-material in early post-natal life led him, as we have seen, to the conclusion that the formation of 1 kilo of mammalian tissue required 4808 Cal. (i.e. total absorption, combustion plus storage), and that in pre-natal life it required about 4000 cal. (2500 for combustion and 1500 for storage). It is evident that the efficiency here is very low, but attention may for the time being be concentrated on the absolute magnitude of the combustion quota. Tangl noticed that Rubner's "law of intra-uterine developmental energy" seemed different from what his own results on the eggs of the lower animals would have led him to expect. Thus to build 1 kilo of silkworm during its embryonic life

| | Calories |
|---|---|
| *U* (Rubner) or Ea. (Tangl) ... ... | 875 |
| *W* (Rubner) or specific energy-content | 1352 |
| | 2227 |

[1] There may also conceivably be a quota of energy used in establishing the O.E.; this quota will form part of the Ea.

are required. This contrasts very markedly with the parallel calculation of Rubner for several mammals (horse, cow, sheep, pig, and dog):

| | Calories |
|---|---|
| $U$ or Ea. ... ... ... ... | 2480 |
| $W$ or specific energy content ... | 1504 |
| | 3984 |

One of the main differences between the two results is that the figures of Tangl were the result of a large amount of experimental work, whereas those of Rubner were approximations calculated on the basis of various more or less doubtful assumptions (see p. 494).

It followed that the efficiencies were divergent. The storage expressed in per cent. of the absorption of nourishment, Rubner's "energetischer Nutzungsquotient" or $\frac{W}{U+W} \times 100$, was, for Rubner's mammalian embryos, on an average 34·3, and for man was as low as 5·2, while, as will have already been noted from Table 121, for the silkworm it was 63·2 and for the chick 67·0. Tangl concluded that in the two latter cases quite other governing processes operate than in the case of mammals, but it is probable that future work will not support Rubner's "law". It is extremely difficult to see why intra-uterine development should be so much less efficient than development within an egg; the statement, indeed, has almost the status of a biological paradox. At the same time, it would be interesting to know what takes place as regards energy exchange in, for instance, an ovo-viviparous selachian.

It may be noted, however, that Rubner's law was found by Tangl to hold quite well for the early post-embryonic life of the silkworm.

| | Experimentally found Calories | Calculated on Rubner's basis Calories |
|---|---|---|
| S.Ea. or $U_t$ ... ... | 11643 | 11010 |
| $W_t$ ... ... ... | 4085 | 5010 |
| | 15728 | 16020 |

It would, therefore, appear that Rubner's law holds in extra-uterine or post-embryonic development only. If this is so, it is possible that the efficiency of the mammalian embryo may be very like that of the non-mammalian embryo, and in any case higher before than after birth.

A number of other workers have occupied themselves with the energy relations of various embryos. Their experiments permit of the

following table, which summarises the average efficiencies at present known.

Table 122.

| | A.E.E. (apparent energetic efficiency) [Synonyms: Rubner's "energetische Nutzungsquotient"; Terroine & Wurmser's "rendement énergétique brut"] | |
|---|---|---|
| CALCULATED | % | Investigators |
| Horse embryo ... ... ... ... | 33·3 | Rubner |
| Cow embryo ... ... ... ... | 33·1 | ,, |
| Sheep embryo ... ... ... ... | 38·2 | ,, |
| Pig embryo ... ... ... ... | 40·0 | ,, |
| Dog embryo ... ... ... ... | 34·9 | ,, |
| Cat embryo ... ... ... ... | 33·0 | ,, |
| Rabbit embryo ... ... ... ... | 27·7 | ,, |
| Average value for mammals ... (N.B. These values were not proved by Rubner to hold for pre-natal life, and he thought the average might there be slightly higher, say, between 38 and 41 %) | 34·3 | |
| Human embryo ... ... ... ... | 5·2 | ,, |
| EXPERIMENTALLY DETERMINED | | |
| Lecithic | | |
| Chick embryo ... ... ... ... | 62·9 | Tangl |
| Chick embryo ... ... ... ... | 67·0 | Tangl and Murray |
| Silkworm embryo ... ... ... | 63·2 | Farkas |
| Minnow embryo ... ... ... | 52·8 | Glaser |
| Frog embryo (to hatching only) ... | 82·0 | Fauré-Fremiet & V. du Streel |
| Frog embryo (to disappearance of external gills only) | 75·5 | Barthélémy & Bonnet |
| Frog embryo (to end of yolk-sac) ... | 51·1 | Fauré-Fremiet & Dragoiu |
| Alecithic | | |
| *Sabellaria* embryo ... ... ... | 97·9 | Fauré-Fremiet |
| *Ascaris* embryo ... ... ... ... | 95·1 | ,, |
| | R.E.E. | |
| EXPERIMENTALLY DETERMINED | | |
| Cow adult ... ... ... ... ... | 55·9 | Kellner & Köhler |
| Pig adult ... ... ... ... ... | 68·0 | Fingerling, Köhler & Reinhardt |
| Mould, *Aspergillus niger* ... ... ... | 70·0 | Terroine & Wurmser |

Several points of interest are to be noted about this table. In the first place, Terroine & Wurmser drew attention to the figure found by Fauré-Fremiet and Vivier du Streel for the development of the frog, namely, 82 per cent., but this figure, though shown in the above table, cannot be compared with the rest, for it only applies to the development that takes place before hatching. It therefore resembles the figure obtained by Barthélémy & Bonnet for the frog, i.e. 75·5

per cent. for development up to the time of disappearance of the external gills. Now it is evident that, in order to get a true efficiency value for any embryo that has a prolonged post-natal yolk-sac or "autophagic" period, the term "finished embryo" can only be applied to the young organism at the end of this time. Since the energy contained in envelopes, excreta, etc., may for the present purpose be regarded as negligible, and since at no time can embryo be separated from yolk, the efficiency is given at any moment by the amount of energy in the larva (i.e. the whole system) expressed in per cent. of the amount of energy present in the whole egg at the beginning. Absorption may be regarded as having taken place instantaneously at fertilisation. Therefore an efficiency of 82 per cent. by hatching simply means that 18 per cent. of the original energy has been lost by combustion, and an efficiency of 75·5 per cent. by the time of disappearance of the external gills means that 24·5 per cent. has been lost by that time. As in each case the "embryo" is partly embryo and partly yolk, these figures do not mean that the earlier periods of development in the frog have higher efficiencies than the later ones; on the contrary, they mean nothing. Happily, Fauré-Fremiet & Dragoiu followed the development of *Rana temporaria* right through to the end of the autophagic period with the bomb calorimeter, and their figure corresponds well enough with that found by Tangl for the chick and with other work.

## 7·4. Real Energetic Efficiency

Terroine & Wurmser, in an important paper on the energy relations of growth, introduced certain new conceptions into the subject. They defined the "rendement énergétique" analogously to the "plastic efficiency coefficient" as:

$$\frac{\text{Energy laid up in the organism}}{\text{Energy in the raw materials at zero hour} - \text{Energy in the raw materials at the end of development}},$$

or

$$\frac{U'}{U - U_R},$$

which is only another way of writing

$$\frac{\text{Energy stored}}{\text{Energy absorbed}} \text{ or } \frac{\text{Energy stored}}{\text{Energy stored} + \text{Energy in solid burnt}},$$

which, multiplied by 100, is the percentage efficiency. This they termed the "rendement énergétique brut", or "apparent energetic efficiency" (A.E.E.). They proceeded to point out, however, that this A.E.E. involves a fallacy, for it does not take into account the basal metabolism—and only if this is done can the "rendement énergétique réel" be computed. Tangl's Ea., as has been pointed out, is only a measure of the total embryonic catabolism. In just the same way the "rendement énergétique brut" fails to allow for the fact that some of the energy absorbed by the embryo is expended in basal metabolism, maintenance energy, "énergie d'entretien", etc., to which the embryo is committed by the mere circumstance of being alive at all. Thus of the energy in the material combusted only a certain fraction ought really to be included in the calculation of the efficiency, for the rest is earmarked for the upkeep of that part of the building already constructed. The "rendement énergétique brut" does not take into account the fact that every cell embarks upon a basal metabolism as soon as it is completed. A calculation of the true growth energy must therefore allow for this, according to the following formula:

$$\frac{\text{Energy laid up in the organism}}{\left(\begin{matrix}\text{Energy in the raw}\\ \text{materials at zero hour}\end{matrix}\right) - \left(\begin{matrix}\text{Energy in the raw materials}\\ \text{at the end of development}\end{matrix} + \begin{matrix}\text{Energy of}\\ \text{maintenance}\end{matrix}\right)},$$

or

$$\frac{U'}{U-(U_R+U_E)}.$$

The denominator is now the energy absorbed for growth and non-basal metabolism only. Armsby also advocated taking the basal metabolism into account. Some doubt may naturally be raised as to whether the usual notions of basal metabolism can be applied to a system so rapidly changing as the embryo. Basal metabolism is that amount of energy given off in the maintenance of a steady state, but can the embryo be considered to be in a steady state even over a short period? However, from another point of view, the embryo has a certain amount of surface, and the minimum production of heat by its cells would be expected to be sufficient to fit in with this; or conversely, it possesses a certain surface corresponding to the minimum heat production of its cells. In either case some approximation to the basal metabolism might perhaps be obtained by calculating what the surface would require. Terroine & Wurmser, in the case of the mould *Aspergillus niger* were enabled to alter its growth-rate

by cultivating it on a medium of abnormal *p*H, but in the case of the chick embryo no such interference with the normal course of events is possible. In 1927 I made some exploratory calculations, using Meeh's formula and Rubner's constant. The calculation could evidently not be exact, because we do not know how these quantities vary during the embryogeny of the chick. The relevant figures are shown in Tables 123 and 124.

In Table 123 the calculated surface of the embryo is obtained according to the formula:

$$S = K\sqrt[3]{W^2},$$

where $S$ is the surface, $K$ Rubner's constant for the chicken, 10·4, and $W$ the weight taken from Murray's data, and Voit's figure

Table 123.

| | Surface of the embryo | | calories evolved in basal metabolism | Total calories evolved (Bohr & Hasselbalch) | |
|---|---|---|---|---|---|
| | Total sq. mm. | Daily increments | Daily increments | Output per day | Daily increments |
| 1 | 2 | 3 | 4 | 5 | 6 |
| Day 0 | — | | | ↑ | |
| 1 | — | — | — | Heat | — |
| 2 | — | — | — | absorbed | — |
| 3 | 100·5 | — | — | ↓ | — |
| 4 | 198 | 98·5 | 92 | 24 | 24 |
| 5 | 380 | 182 | 172 | 48 | 36 |
| 6 | 586 | 206 | 194 | 84 | 31 |
| 7 | 847 | 261 | 246 | 115 | 36 |
| 8 | 1,170 | 323 | 305 | 151 | 49 |
| 9 | 1,550 | 380 | 358 | 200 | 76 |
| 10 | 2,000 | 450 | 424 | 276 | 120 |
| 11 | 2,510 | 510 | 481 | 396 | 156 |
| 12 | 3,080 | 570 | 537 | 552 | 228 |
| 13 | 3,750 | 670 | 632 | 780 | 221 |
| 14 | 4,490 | 740 | 698 | 1001 | 239 |
| 15 | 5,290 | 800 | 754 | 1240 | 220 |
| 16 | 6,150 | 860 | 811 | — | — |
| 17 | 7,130 | — | — | 1460 | 250 |
| 18 | 8,170 | 980 | 924 | 1710 | 250 |
| 19 | 9,280 | 1,040 | 981 | 1960 | 200 |
| 20 | 10,700 | 1,110 | 1050 | 2160 | — |
| | | 1,420 | 1350 | | |
| | Total | 10,599 | | | |

of 0·943 cal. per sq. mm. surface for the chick's basal metabolism is accepted. Col. 4 represents the quantity of inevitable loss on the weight of embryo formed each day. Now if the number of cal. evolved

as measured by Bohr & Hasselbalch in each period of 24 hours is compared with the heat production calculated from the oxygen consumption (Col. 9, Table 124), it will be seen that the agreement is fair, though the experimental is always rather lower than the calculated value. The fact that the calculated value assumes fat only to be burnt would not entirely account for this. Returning to Table 123, however, it can at once be seen that the basal metabolism invariably exceeds the total amount of heat evolved. Thus there is not enough heat eliminated to account for the quantity that ought to be produced in maintenance energy alone. The basal metabolism as here calculated must be far too high, for if all the increments are added up the result is 61,000 calories, or about four times as much as the total energy known to be lost by combustion. We must therefore suppose either that the surface formula does not hold in embryonic life or that the high temperature (37°) in which development proceeds leads to a lower basal metabolism than would be expected. Lusk says that the minimum requirement for energy is seen to be present when the fasting organism is surrounded by an atmosphere having a temperature of 30 to 35°. Most important of all, however, is the probability that Rubner's constant for the chick does not hold for the embryonic chick. It is quiescent, its muscles have no tonus or very little, its respiratory muscles are inactive, and its heart alone is constantly requiring a supply of energy. Since the metabolism is proportional to the superficial area of the animal, it may well be asked what is happening in an embryo at the minute stage when its percentage growth-rate is 1400 (Schmalhausen).

## 7·5. Apparent Energetic Efficiency

Evidently it is not possible to calculate the "rendement énergétique réel" (R.E.E.) for the chick. But Terroine & Wurmser pointed out that some of the discrepancy existing between Tangl and Rubner might be removed if it were known. For the growing cow the R.E.E. was calculated by Kellner & Köhler, and for the growing pig by Fingerling, Köhler & Reinhardt. They estimated the magnitudes of (*a*) the energy stored in the organism following the addition to a fundamental ration of a given foodstuff in known quantity, calculated on the basis of carbon and nitrogen balance and formation of tissue; (*b*) the total energy catabolised measured by indirect calorimetry, and (*c*) the "énergie d'entretien"

computed from the surface law. The result was that the efficiency rose, giving:

| | R.E.E. % | Investigators |
|---|---|---|
| Cow | | |
| Starch ... ... | 58·9 | Kellner & Köhler |
| Gluten ... ... | 45·2 | ,, ,, |
| Oil ... ... ... | 56·3 | ,, ,, |
| Cellulose ... ... | 63·1 | ,, ,, |
| Pig | | |
| Starch ... ... | 84·0 | Fingerling, Köhler & |
| Cellulose ... ... | 52·0 | Reinhardt |

"Ces données", said Terroine & Wurmser, "rentrent parmi les plus intéressantes actuellement possédés. On ne manquera pas de constater qu'une fois prise la précaution d'écarter la dépense d'entretien, si élevée chez un homeotherme, on aboutit à des chiffres tres voisins de ceux de Tangl, Farkas, et Glaser, pour le développement de l'œuf." At first sight this correspondence is fallacious, for we are comparing the R.E.E. of the two mammals with the A.E.E. of the various eggs studied. Nevertheless, the calculation given above shows that the basal metabolism of the chick embryo is probably many times lower than might be supposed, so that the R.E.E. would differ little from the A.E.E. Furthermore, as Terroine & Wurmser point out, the eggs studied are mostly those of poikilothermic animals, whose basal metabolism is known to be very low, and, as for the chick, we know that, for the greater part of its pre-natal life, it behaves as a cold-blooded animal. Rubner's low mammalian values, then, when corrected for basal metabolism, would approach the values of Tangl and his associates, and we shall probably not be far wrong if we assess the R.E.E. of all embryos, mammalian as well as non-mammalian, at about 66 per cent.

The average value of the A.E.E. will presumably vary with varying conditions. Is it affected by temperature? The only answer to the question is contained in the papers of Barthélémy & Bonnet, whose experiments were conclusive. These investigators, as we have already seen, studied calorimetrically the development of the frog's egg (*Rana fusca*) up to the disappearance of the external gills, and caused the development to take place at different temperatures. Their results were as follows:

| Temperature °C. | Time of development days | Efficiency (A.E.E.) i.e. $\frac{\text{Energy in } n \text{ embryos}}{\text{Energy in } n \text{ eggs}}$ | |
|---|---|---|---|
| 9 | 30 | 74–78 | Average 75 |
| 11 | 22 | 71–76 | ,, 73 |
| 14 | 20 | 71–84 | ,, 75 |
| 21 | 8 | 70–82 | ,, 75 |

Evidently the temperature exercises no effect on the proportion of energy used for storage and that used for combustion. More or less analogous results had previously been found for the development of *Proteus vulgaris* by Rubner, for muscular contraction by Hill, for the growth of *Sterigmatocystis nigra* by Terroine & Wurmser, and for the germination of seeds by Terroine, Bonnet & Joessel. The invariability of the A.E.E. of embryonic growth would seem, then, to be a special case of a general biological law.

The next question which arises is whether the efficiency varies from time to time during the development of the embryo; we have already seen that the P.E.C. shows such a variation. Table 124 gives the calculations for determining this (Needham). The increments of calories, i.e. the amounts of potential energy stored in the embryonic body each day, are checked against the energy present in the extra-embryonic part of the egg as determined with the bomb calorimeter by Tangl & von Mituch. It will be seen that the rest of the egg loses, in addition to combustion, 250 cal. between the 8th and the 9th days, while the embryo gains 232; a sufficient agreement. The figure of 34,000 cal. seen at the bottom of Col. 4 representing the number of calories contained in the finished embryo agrees sufficiently well with the value given by Tangl of 32,000; the latter was measured directly, the former was obtained by the addition of all the increments.

Cols. 6 to 9 give the figures relating to the energy lost in combustion. This is obtained, assuming that 100 per cent. instead of the true 92 per cent. of the total solid burnt is fat, and that 1 gm. of fat produces on its combustion 9300 cal. The total of this column amounts to 17,000 cal., not very far from the 16,500 cal., the Ea. of Tangl. In Col. 10 Tangl's values for Col. 9 are given, and it may be noticed that they are close to the newer ones. An error exists here owing to the fact that no account has been taken of the energy left behind in incompletely combusted materials, but as the chief of these is uric acid, and—using the data of Stohmann & Langbein for the calorific value of uric acid, 2750 cal. per gm. mol.—the calories locked up in this way only amount to 16 on the 10th day, or much less than 1 per cent. of the total combusted; this error is negligible. Finally Col. 11 shows the A.E.E., which is diagrammatically represented in Fig. 260. Starting at a low level, it slowly rises, gaining in speed till at the 14th day it is rising rapidly, but soon afterwards it falls off. The value for the whole of development works out at 66·5, which is exactly what

## Table 124.

| | | Energy stored in the embryo | | | Energy in whole egg contents (Tangl) (smoothed) | Solid lost by combustion (calculated from oxygen consumption by Murray) | | Inter-diurnal averages | Calorific value of Col. 8 calculated as fat (calories) | Col. 9 according to Tangl | $\frac{U'}{U-U}$ or A.E.E. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (Murray) calorific value per gram dry weight (cal.) | Actual value in calories | Increments | | Milligrams per gram dry weight | Actual milligrams each day | | | | |
| 1 | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Day | 0 | — | — | — | — | — | — | — | — | — | — |
| | 1 | — | — | — | — | — | — | — | — | — | — |
| | 2 | — | — | — | — | — | — | — | — | — | — |
| | 3 | 5080 | 7·4 | 16·6 | — | 990 | 1·4 | 2·4 | 22 | — | 43·0 |
| | 4 | 5095 | 24 | 36 | — | 740 | 3·49 | 4·8 | 45 | — | 44·4 |
| | 5 | 5110 | 60 | 61 | — | 520 | 6·11 | 8·3 | 77 | — | 44·2 |
| | 6 | 5125 | 121 | 101 | — | 444 | 10·5 | 13·0 | 121 | — | 45·5 |
| | 7 | 5150 | 222 | 160 | 88250 | 364 | 15·6 | 19·3 | 180 | — | 47·1 |
| | 8 | 5180 | 382 | 232 | 88000 | 311 | 23·0 | 27·9 | 260 | 250 | 47·2 |
| | 9 | 5200 | 614 | 358 | — | 278 | 32·8 | 39·5 | 367 | — | 49·4 |
| | 10 | 5220 | 972 | 538 | — | 248 | 46·2 | 54·7 | 509 | — | 51·4 |
| | 11 | 5240 | 1510 | 860 | — | 219 | 63·2 | 73·6 | 684 | — | 55·9 |
| | 12 | 5270 | 2370 | 1340 | — | 187 | 84·0 | 97·0 | 902 | — | 59·8 |
| | 13 | 5380 | 3710 | 2340 | 85700 | 159 | 110·0 | 125·0 | 1160 | 1000 | 66·9 |
| | 14 | 5500 | 6050 | 3510 | 84700 | 128 | 141·0 | 156·0 | 1450 | — | 70·7 |
| | 15 | 5710 | 9560 | 4240 | — | 103 | 172·0 | 188·0 | 1750 | — | 70·8 |
| | 16 | 5860 | 13800 | 4600 | — | 87 | 205·0 | 218·0 | 2030 | — | 69·4 |
| | 17 | 5970 | 18400 | 5000 | 81000 | 75 | 232·0 | 242·0 | 2250 | 2000 | 68·9 |
| | 18 | 6030 | 23400 | 5200 | 79000 | 65 | 253·0 | 258·0 | 2400 | — | 68·4 |
| | 19 | 6070 | 28600 | 5600 | — | 56 | 264·0 | 272·0 | 2530 | — | 68·9 |
| | 20 | 6110 | 34200 | | — | 50 | 280·0 | | | | |
| Total | | — | — | 34193 | — | — | — | — | 17237 | — | 66·5 |

Tangl found. Since the basal metabolism is included in this estimate, and since that might naturally be expected to be high in the early stages when the embryo is very minute, and has a large surface in proportion to its size, one can understand that the efficiency, the A.E.E., would then be very low.

Another way of interpreting Fig. 260 would be by the recapitulation theory. Perhaps the most striking chemical attribute of the bacteria and yeasts is their high energy turnover: Horace Brown, for example, showed that a yeast cell would ferment its own weight of maltose at 30° C. in 2·2 hours, and at 40° C. in 1·3 hours. This metabolic level would be about 100 times as high as that of an adult man. And Haacke has calculated that certain lactose fermenting bacilli destroy from 178 to 14,980 times their own weight of lactose per hour. Parallel with this furious onslaught on the nutrient material of their environment goes a very low efficiency[1], figures for which are available in a number of papers:

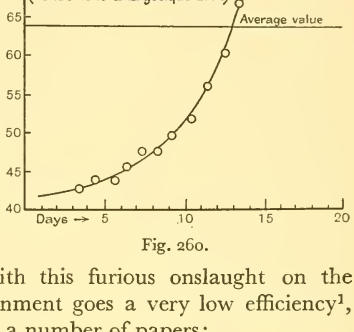


Fig. 260.

| Investigator | Organism | Efficiency (%) |
|---|---|---|
| Stephenson & Whetham | *Timothy Grass Bacillus* | 27·0 |
| Becking & Parks ... | *Nitrobacter* | 7·9 |
| ,, ... | *Nitrosomonas* | 5·9 |
| ,, ... | *B. methanicus* | 15·1 |
| Ruhland ... ... ... | *B. pycnoticus* | 20·5 |
| Waksman & Starkey | *Thiobacillus thiooxidans* | 6·2 |
| Beijerinck ... ... ... | *Thiobacillus denitrificans* | 8·7 |

In discussing these facts Stephenson suggests that the breakdown of a substance such as sugar by the yeast-cell or a bacillus is conditioned mainly by the concentrations of cells (enzymes) and substrate, irrespective of whether the cells can benefit by the energy liberated. Thus the energy liberated by micro-organisms would be no measure of their metabolic needs but simply the result of unprotected enzymes acting upon the appropriate pabulum. "If such a view be correct"

[1] But it must be understood that micro-organisms have really no definite efficiency; it varies according to their environment, and they have no means of adjusting it. Bacteria "killed" by ultra-violet light continue to oxidise at almost the normal rate, though incapable of growing, and in yeast cultures fermentation and growth are quite dissociable. When growth ceases the efficiency is nil, but in certain conditions it may be as high as 59 % (Terroine & Wurmser on moulds).

says Stephenson "one may regard the evolution of the metazoal organism as involving a process whereby the energy liberated in chemical activity, which in a microbe runs to waste, is so organised and disciplined that it is liberated when and where it can subserve function, such as muscular work or maintenance of temperature; apart from such organised expenditure the liberation of energy in the higher animal is cut down to a minimum represented by its basal metabolism or energy of maintenance." She left the question open as to whether this latter quota might be, even in mammals, an expenditure which the animal was unable to prevent or, conversely, of some deeper significance. Her picture of the gradual increase of organisation in evolution, is one of much interest and may apply also to the ontogenetic passage from low to high efficiency seen in Fig. 260. Possibly the chick embryo in its earliest stages may resemble the micro-organisms in being unable to keep its enzymes apart from its substrates, although it may be noted that its efficiency is not lower than 40 per cent. at the worst[1].

A third way of considering the phenomenon of rising efficiency is that of Terroine and his collaborators. In their studies of the germination of seeds (see Appendix III) they found that—roughly speaking—the A.E.E. was highest when the reserves were mainly in the form of carbohydrate, mediocre when the reserves were in the form of fat, and lowest when they were made up of protein. Now the seedling itself, which corresponds to the developing embryo, may be considered as made up almost entirely of cellulose, i.e. carbohydrate, and Terroine and his colleagues therefore concluded that the A.E.E. varied with the nature of the food-materials, i.e. was a measure of the degree of chemical difference between the reserve materials and the finished structure. The least wastage occurred when carbohydrate was used, more when fat was used, and most when protein was used. In their work on germination it was assumed (for the sake of argument) that the composition of the seedling and the reserves was throughout the same, and this was justifiable enough as the A.E.E., estimated at various moments in germination, was always found to be the same. But in the bird's egg, the composition of the embryo does not remain the same; profound changes are taking place all the time in its

[1] Cf. the condition seen in the unfertilised echinoderm egg (p. 626). But the embryo in the early stages seems not only to combust an excess of nutritive material, but also to fail to retain properly those building-stones which it does not combust, judging from the high proportion of amino-acid nitrogen in the allantoic liquid at that time (see p. 1096). Could this also be of ancestral significance? (see Table 163).

constituent substances and their balance, nor do the yolk and white remain chemically constant. Perhaps the embryo at the 5th day of development has much more to do to whatever it is absorbing to turn it into itself than has the embryo of the 15th day, and consequently the wastage is greater.

This seems at first sight to be in contradiction with the facts known about the "white yolk" (see p. 286) which, as Spohn & Riddle's work showed, resembles the embryonic tissue much more than it does the yellow yolk. But it is reasonable to suppose that this phase would be passed through by the 5th day, at which time the A.E.E. curve begins, and we might predict that when the efficiency of the earlier stages is known, it will turn out to be higher, perhaps as much as 60 per cent. The A.E.E. curve would then become trough-shaped, like the P.E.C. curve (see Fig. 254).

The argument due to Terroine would thus be that in the earlier stages the raw materials are more unlike the embryonic body in composition than they are later. An interesting calculation which shows that this is to some extent true is shown in Table 125, where first of all the absolute amounts of the three main cell-constituents, carbohydrate, protein and fat, are set down, both for the embryo and for the raw materials, i.e. for the remainder of the egg. Then these figures are expressed as percentages of the sum of the three in each case and given in Cols. 8 to 13, so that we have side by side the variations in balance of the three classes of substance throughout development.

*Difference between the two figures (embryo and remainder) in Table 125.*

| | At the beginning (4th day) | At the end (20th day) |
|---|---|---|
| Carbohydrate (Cols. 8, 11) ... ... | 2·3 | 0·3 |
| Protein (Cols. 9, 12) ... ... ... | 29·5 | 9·5 |
| Fat (Cols. 10, 13) ... ... ... | 31·8 | 9·8 |

It can hardly be a coincidence that all three should work out like this, but too much emphasis must not be laid on the calculation in view of the fact that the sets of figures used for it are derived from the results of several workers using not very homogeneous material. A more serious shortcoming of such a calculation is that it assumes that the embryo must be absorbing throughout an aliquot part of the raw materials, although we may be reasonably certain that this never happens at all. And again the efficiency curve involves qualitative

## Table 125.

| | Absolute amounts of substances (mgm.) | | | | | | Values in % of the sum of the three | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | In embryo | | | In raw material | | | In embryo | | | In raw material | | |
| | Carbohydrate | Protein | Fat | Carbohydrate | Protein | Fat | Carbohydrate | Protein | Fat | Carbohydrate | Protein | Fat |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| | Needham | Murray and Needham | Murray and Idzumi | Needham | Sakuragi | Sakuragi and Idzumi | | | | | | |
| Day 0 | — | — | — | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | — | — | — | — | — | — | 66·0* | 33·0* |
| 2 | — | — | — | — | — | — | — | — | — | — | — | — |
| 3 | — | — | — | — | — | — | — | — | — | — | — | — |
| 4 | 0·166 | 3·46 | 0·67 | 193 | 6140 | 5599 | 3·9 | 81·0 | 15·1 | 1·6 | 51·5 | 46·9 |
| 5 | 0·33 | 6·9 | 1·85 | 169 | 6110 | 5598 | 3·6 | 75·0 | 21·4 | 1·4 | 51·5 | 47·1 |
| 6 | 0·54 | 12·5 | 3·49 | 152 | 6070 | 5596 | 3·3 | 76·0 | 20·7 | 1·3 | 51·1 | 47·6 |
| 7 | 0·84 | 23·5 | 6·53 | 141 | 5970 | 5594 | 2·7 | 76·4 | 20·9 | 1·2 | 51·0 | 47·8 |
| 8 | 1·31 | 42·6 | 11·51 | 144 | 5900 | 5569 | 2·4 | 77·0 | 20·6 | 1·2 | 50·8 | 48·0 |
| 9 | 2·08 | 71·0 | 19·01 | 191 | 5800 | 5511 | 2·3 | 77·0 | 20·7 | 1·7 | 50·4 | 47·9 |
| 10 | 3·17 | 111 | 36·7 | 230 | 5690 | 5413 | 2·1 | 73·7 | 24·2 | 2·0 | 50·2 | 47·8 |
| 11 | 4·69 | 174 | 49·1 | 230 | 5580 | 5301 | 2·1 | 76·5 | 21·4 | 2·1 | 50·1 | 47·8 |
| 12 | 7·57 | 272 | 78·6 | 214 | 5400 | 5172 | 2·1 | 76·0 | 21·9 | 2·0 | 50·1 | 47·9 |
| 13 | 11·23 | 419 | 125·7 | 193 | 5190 | 5004 | 2·0 | 75·5 | 22·5 | 1·8 | 50·0 | 48·2 |
| 14 | 17·84 | 684 | 207·7 | 175 | 4840 | 4802 | 1·9 | 75·4 | 22·7 | 1·8 | 49·3 | 48·9 |
| 15 | 25·8 | 1006 | 333 | 164 | 4390 | 4527 | 1·8 | 73·5 | 24·7 | 1·8 | 48·4 | 49·8 |
| 16 | 36·8 | 1500 | 498 | 139 | 3880 | 4172 | 1·8 | 74·0 | 24·2 | 1·7 | 47·5 | 50·8 |
| 17 | 52·10 | 1940 | 701 | 128 | 3340 | 3799 | 1·9 | 72·2 | 25·9 | 1·8 | 45·9 | 52·3 |
| 18 | 68·5 | 2340 | 979 | 112 | 2830 | 3271 | 2·0 | 69·0 | 28·0 | 1·8 | 45·5 | 52·7 |
| 19 | 87·4 | 2680 | 1335 | 80 | 2380 | 2575 | 2·1 | 65·0 | 32·9 | 1·6 | 47·3 | 51·1 |
| 20 | 107·2 | 2970 | 1764 | 70 | 1910 | 1686 | 2·2 | 61·5 | 36·3 | 1·9 | 52·0 | 46·1 |

* White yolk.

differences between the raw materials and the embryo itself, so that not only is the balance between carbohydrate and protein changing through development, but also the balance of integral portions of the carbohydrate fraction. Nevertheless it is interesting that the rough calculation of Table 125 does confirm the conception of an increasing similarity between the embryo and its raw materials with age, and it would be extremely valuable to collect data for a profounder examination of the problem not only in the chick but in many different animals.

It may be noted also that, if the embryo continued to behave as wastefully all through incubation as it does in the beginning, there would not be nearly enough energy in the egg to provide for it, unless the egg were increased to about twice its present size. Even then there would be no reserve yolk at hatching.

Since the A.E.E. rises with age, it resembles the percentage of total solids, the percentage of fat, the latent period of growth in tissue culture fragments, the total metabolism and the rate of the heart beat; a miscellaneous collection of factors. But, having Murray's rule in mind, and remembering that embryonic development is symmetrically diphasic in character, we may enquire whether it moves rapidly at first, then slowly, like the growth-rate, or slowly at first, then rapidly, like the metabolic rate. Evidently the rise in A.E.E. resembles the fall in metabolic rate. Thus it would seem as if the furious intensity of combustion with which the embryo begins its life was associated with great wastefulness, while later on greater economy would accompany greater frugality. The calorific value of the embryonic tissue also rises during development, and Murray's graph (Fig. 256) shows that it goes up in a curve shaped rather like that for the A.E.E. Thus the richer in potential energy the embryonic body becomes per unit weight, the more efficient is the transfer of energy from the yolk and white.

Though the curves for P.E.C. and A.E.E. are different, it is interesting to find that the average P.E.C. for all development is 0·68, while the average A.E.E. is 66 per cent. Out of 100 gm. of solid presented to it, the embryo can store 68; out of 100 cal. presented to it, the embryo can store 66.

The conclusion that the A.E.E. of the chick embryo increases with age, and that, in the early stages of development, storage of energy is very inefficient, is in agreement with the views of Armsby. That it rises, as we have seen, to approximately 70 per cent. at hatching, is

interesting in view of the efficiencies found by other workers on the early post-natal life of mammals, thus:

| | A.E.E. | R.E.E. | |
|---|---|---|---|
| Man ... ... | 84·08 | 73·10 | Rubner & Heubner |
| Pig ... ... | 83·99 | 70·31 | Wilson |
| Calf ... ... | 86·18 | 73·77 | Soxhlet |

Armsby stated that in later post-natal life the efficiencies were higher still, but though he calculated them from Kern & Wattenberg's data on the sheep, Tschirwinski's data on the pig, and Armsby & Fries' data on the cow, he did not give any actual values. From a general point of view, therefore, it is probable that as more data come to light it will be found that the efficiency of the organism considered as a machine for storing energy rises from fertilisation to death. Nothing is known about the rapidity with which the adult level of efficiency is reached, but Armsby & Fries considered that this would probably occur not long after weaning (see also Brody).

A comparison may be made between the embryo and other engines. Its business is to store as much energy as is given it with as little loss as possible. The object of the steam engine is to produce as much mechanical work from the energy given it with as little loss as possible. The efficiency of this process is not great; in the locomotive engine, which is notoriously wasteful, it may not exceed 15 per cent. Wimperis and Bird give 25 per cent. for the gas engine with suction producer, and the best recorded efficiency for a Diesel engine with high maximum pressure is 40 per cent. But a much better comparison is between the embryo and the electric accumulator, for this does not alter the form of the energy passing through it. Cooper's average estimate is that an electric accumulator will give back 74 per cent. of the energy put into it, and another figure (Davidge & Hutchinson) is 70 per cent. It is interesting that the average A.E.E. of developing embryos should be of the same order.

## 7·6. Synthetic Energetic Efficiency

Now Terroine & Wurmser's formula for calculating the R.E.E. was

$$\frac{\text{Energy stored in the embryo}}{\left(\begin{matrix}\text{Energy in raw materials}\\ \text{at zero hour}\end{matrix}\right) - \left(\begin{matrix}\text{Energy in unused}\\ \text{raw materials}\end{matrix} + \begin{matrix}\text{Energy in solid burned}\\ \text{for basal metabolism}\end{matrix}\right)},$$

or, in their notation:

$$\frac{U'}{U - (U_R + U_E)}.$$

This is perfectly satisfactory so long as we only consider complete combustions to carbon dioxide and water. Rapkine, however, pointed out that the developing organism may have at its disposal other sources of energy, for endothermic reactions are known to occur *in vivo*, which raise the chemical potential of their products. Confusion arose here owing to the fact that some workers used the term "energy sources" to apply to the solids burned to give the heat lost from the egg, while others used it to apply only to those reactions, whatever they may be, which gave the energy of organisation, or O.E. Bohr & Hasselbalch showed finally that of the total solid lost not more than 4 per cent. can participate in the O.E., but when we take into account the energy not furnished by complete combustions it is legitimate to suppose that a larger proportion of the total energy turnover may be used for O.E. We cannot expect to find this, of course, by bomb calorimetry, for the organisation is destroyed by drying. Rapkine focused attention upon coupled and spontaneous endothermic reactions, and considered that their existence in the embryo explained *inter alia* (1) the atypical respiratory quotients which he had himself observed in echinoderm eggs (see p. 648), (2) the low calorific quotients of Meyerhof (see p. 651), (3) the initial heat absorption in Bohr & Hasselbalch's measurements (see p. 704), and (4) the synthesis of fatty substances which proceeds in many eggs. Applying these ideas to the efficiency formula, Rapkine suggested that the numerator $U'$ (calories in unit weight of finished embryo) should be replaced by $U'$ minus the energy contained in an exactly equivalent weight of original raw material. This would represent the elevation of calorific value which has gone on during development:

$$\frac{U' - x}{U - (U_R + U_E)}.$$

It can be seen at once that this will give an efficiency of a very low order, but not altogether comparable with those which have already been discussed, such as the A.E.E. and the R.E.E. For what they measure is the relation between the energy in the substance stored in the embryo or transformed into its tissues, on the one hand, and the energy absorbed by the embryo from the raw materials, on the other hand, either allowing for the basal metabolism or not allowing for it. Rapkine's efficiency coefficient, on the contrary, measures the relation between the energy furnished to the embryo from coupled reactions, etc. (energy which would have been dissi-

pated as heat if the endothermic processes had not caught and held it), on the one hand, and the energy absorbed by the embryo from the raw materials, on the other hand, allowing for the basal metabolic requirements. It thus has to do, not with energy storage as a whole, but with the storage of energy from a particular source, i.e. coupled reactions with one endothermic component[1]. During the absorption of 100 cal. of energy, 66 will be stored and 33 lost, but only 9, say, out of that 66 will be saved from the loss by the endothermic processes. Rapkine's coefficient may, therefore, be called the S.E.E. (synthetic energetic efficiency). As a concrete example, in the case of the chick, the values as averaged from many observations can be read off from Fig. 259, and the usual fraction for R.E.E. is

$$\frac{37{\cdot}5}{(86{\cdot}85) - (26{\cdot}4 + 17\ (\text{say}))} = 70{\cdot}9 \text{ per cent.}$$

For the S.E.E. the numerator would at first sight seem to be a minus quantity, for in Murray's work, for instance, the calorific value of 1 gm. of dry unincubated mixed yolk and white was 6·94 Cal. and that of 1 gm. of dry finished embryo was 6·2 Cal., so that no increase in specific energy-content would appear to have taken place. However, the finished embryo is not comparable with the unincubated yolk and white, for a notable proportion of the fat in the latter disappears by combustion, and some of it is left behind as spare yolk at hatching. The following calculation is therefore required to give the energy value of an amount of yolk and white roughly comparable with the finished embryo:

The finished embryo of the chick weighs 6·00 gm. dry weight and has in it 37·5 Cal. i.e. 6·2 Cal. per gram dry weight.

| | | | |
|---|---|---|---|
| The egg at the beginning of development has inside it 12·45 gm. dry weight and 86·85 Cal., i.e. 6·94 Cal. per gram dry weight ... | | 86·85 Cal. = | 100 % |
| For combustion 2·5 gm. fat are used, which at 9·3 Cal. per gram is 23·25 Cal. ... ... ... | | 23·25 Cal. = | 26·4 % |
| For yolk unused at the end of development, i.e. about 4·75 gm. dry weight of which 40·5 % is fat and 50 % protein, i.e.: | | | |
| For fat ... | 17·8 Cal. | | |
| For protein ... | 11·4 Cal. | | |
| | 29·2 Cal. ... ... | 29·20 Cal. = | 30·4 % |
| | | 52·45 Cal. | |
| | | 86·85 – 52·45=34·40 Cal. | |

[1] It should be noted that this is the only kind of energy-storage which will raise the chemical potential of the embryonic body.

An amount of yolk and white, therefore, equivalent to the finished embryo has 34·4 Cal. as against its 37·5 Cal. But from this 34·4 Cal. must be subtracted a correction for the skeletal system of the finished chick, which contains very little energy.

The 37·5 Cal. is the heat contained in, not 6·00 gm. dry weight but 4·5 gm. dry weight, for the bones weigh 1·5 gm. dry weight (Tangl), i.e. 75 % of 34·4 Cal. will give the energy of an amount of yolk and white equivalent to the finished embryo = 25·7 Cal.

Then $37{\cdot}5 - 25{\cdot}7 = 11{\cdot}8$ = energy stored in embryo by the endothermic components of coupled reactions, i.e. increase of chemical potential.

This gives for the S.E.E.:

$$\frac{U' - x}{U - (U_R + U_E)} \text{ or } \frac{37{\cdot}5 - 25{\cdot}7}{86{\cdot}85 - (26{\cdot}4 + 17\ (\text{say}))} \times 100 = 27{\cdot}15 \text{ per cent.}$$

But this calculation is only of illustrative significance, for the data are taken from various different sets of material. It is evident, nevertheless, that the S.E.E. will always work out at a very low level, certainly under 30 per cent.

Wurmser, independently following a like train of thought, found 26 per cent., instead of Terroine & Wurmser's 70 per cent., for the growth of *Aspergillus nigra*. Rapkine suggests that it might be possible to discover what these coupled reactions are which store extra energy in the embryo and provide most of the O.E., by placing various hydrogen donators in the presence of embryonic tissues at different stages of development and following electrometrically their dehydrogenation. Cahn gives the following argument to show that they do not interfere much in the formation of the protein part of the embryo:

| | |
|---|---|
| 1 gm. of the total proteins of the whole egg on the 9th day has a calorific value of 4·91 Cal. and therefore for 5·846 gm. ... | 28·700 Cal. |
| 1 gm. of the proteins of the remainder of the egg on the 21st day has a calorific value of 4·72 Cal. and therefore for 2·36 gm. | 11·150 Cal. |
| 1 gm. of the proteins of the embryo on the 21st day has a calorific value of 5·28 Cal. and therefore for 3·22 gm. ... ... | 17·000 Cal. |
| | 28·150 Cal. |

(The figures are Cahn's.) So there is a difference of 550 cal. and as, judging from Fridericia's uric acid figures (which are probably too high), about 135 mgm. of protein are combusted and therefore 500 cal. liberated, the balance was regarded by Cahn as exact enough to allow of the conclusion that there was no measurable O.E. in this case.

Attention may be drawn to the similarity shown in Fig. 259 between the fraction of energy contributed to the embryo by synthetic processes and the O.E. Both of these are, of course, quite

hypothetical as regards magnitude. At present there does not seem to be any way of measuring the O.E. directly, but a word may be said about certain attempts at doing so which have been made in the past.

In the first place, it has been argued that if any energy is bound up with structure or organisation, this energy ought to be liberated when the structure or organisation is destroyed. Accordingly from time to time attempts have been made to discover the thermal effect of death, and of these the most recent and successful is that of Lepeschkin who killed yeast-cells in various ways as instantaneously as possible and measured the extra heat eliminated. It worked out at 2 cal. per gram of dry weight. If this energy may be in any way regarded as O.E. or the equivalent of O.E. then it might be expected to be rather greater in a metazoal, i.e. more heterogeneous, population of cells, such as the avian embryo, but even so could not exceed 20 gm. cal. in the finished chick. This amount would appear as a line rather than a solid block in Fig. 259. Rychlevska has also some relevant information obtained by combusting tissues without preliminary drying.

Secondly, there is the notion that the O.E. could be found by estimating the work required to stop its formation. Under the head of osmotic pressure, we have already had occasion to examine the work of Spaulding and of Vlès & Dragoiu on the "travail osmotique d'arrêt" of cell-cleavage. Arguing in exactly the same way, Fauré-Fremiet, Henri & Wurmser determined the amount of radiant energy required to stop the segmentation of *Ascaris* eggs. An exposure to ultra-violet light of wave-length 2800 Å. sufficed. Thus $12 \cdot 10^5$ ergs per square centimetre were required to stop development, and the receiving surface of the egg was $2 \cdot 3 \cdot 10^{-5}$ sq. cm., so that the quantity of energy received by an egg and sufficient to block its development was equal to:

$$12 \cdot 10^5 \times 2 \cdot 3 \cdot 10^{-5} = 12 \times 2 \cdot 3 = 27 \text{ ergs.}$$

Fauré-Fremiet contrasted this value with the value for work done during the whole of development calculated from his Ea. results. Thus 280 cal. $\times$ $4 \cdot 18 \cdot 10^9$ ergs = $11 \cdot 6 \cdot 10^9$ ergs per gram dry weight, and, as the specific gravity of *Ascaris* eggs is 1·08 and their diameter $82\mu$, the average weight of one egg must be $3 \cdot 11 \cdot 10^{-7}$ gm. During the development of one *Ascaris* embryo, then, $503 \cdot 10^{-7}$ cal. or 2100

ergs are used up. But the segmentation period is only a comparatively short part of the whole period, so that, allowing for this, a value of 39 ergs was obtained, i.e. of the same order as that found by blocking the first cleavage with ultra-violet light. It is evident, however, that these calculations can tell us nothing at all about the O.E., for the processes of embryonic development are not reversible, and as there is no equilibrium point there can be no sense in determining the amount of energy required to stop the processes from going on.

### 7·7. The Sources of the Energy Lost from the Egg

Although practically nothing is known about the origin of the energy which forms the O.E., a good deal can be said about the origin of the energy which furnishes the Ea., or total catabolism of embryonic life. As we have already seen in the section on the general metabolism of the embryo, there is some reason for supposing that, during pre-natal life, the principal constituents of the embryonic body reach their maxima in a definite succession, i.e. inorganic substances, carbohydrate, protein, fat. The possible significance of this from a general point of view was there discussed, and comparison was made between these data and the varying intensities of absorption of these important components. But an ontogenetic succession of carbohydrate, protein, and fat makes its appearance not only when these three substances are considered as elements in the architecture of the embryo but also when they are considered as reserves of energy for the Ea., fuel-sources for purposes of combustion. Considered in this way, it is certain that in the case of the chick, for instance, carbohydrate attains its highest point in the first week, protein in the second and fat in the third.

For a long time it was generally considered that fatty substances were the sole sources of energy for the developing chick embryo. This view, which was satisfactory only as the roughest of approximations, grew naturally out of the early researches of Pott; Liebermann; and Tangl & von Mituch. Estimations of the fat in the embryo and that in the rest of the egg showed that a good deal had been lost in transit, actually from 2·1 to 2·76 gm. per egg on an average. Calculation demonstrated, as we have already seen, that this figure corresponded satisfactorily with the carbon dioxide evolved throughout incubation, and the heat produced during the same time. For

example, Liebermann had shown that the fat of the egg contained 71·67 per cent. of carbon, and the amount used per egg Tangl & von Mituch found to be on an average 2·11 gm. According to Hasselbalch, the total amount of carbon dioxide produced during incubation amounted to 5·939 gm. From this they calculated the amount of fat which ought to have been used, and the result was 2·26 gm., which agreed moderately well with Liebermann's figure, and with one which they themselves found, 2·76 gm. Another line of investigation which gave support to this view was the work of Bohr & Hasselbalch on the respiratory quotient of the hen's egg, which gave from the 7th day onwards a figure of 0·73. The following curious argument occurs in Liebig's *Animal Chemistry* of 1842, and shows once more the same point of view. "The egg of a fowl can be shewn to contain no other nitrogenised compound except albumen. The albumen of the yolk is identical with that of the white, the yolk contains besides only a yellow fat in which cholesterine and iron may be detected. Yet we see in the process of incubation, during which no food and no foreign matter except the oxygen of the air is introduced or can take part in the development of the animal, that out of the albumen feathers, claws, globules of the blood, fibrine, membrane and cellular tissue, arteries and veins are produced. The fat of the yolk may have contributed to a certain extent to the formation of the nerves and the brain, but the carbon of this fat cannot have been employed to produce the organised tissues in which vitality resides because the albumen of the white and yolk already contains for the quantity of N present, exactly the proportion of carbon required for the formation of these tissues."

It might always have been doubted, however, that fat was the only important source of energy: there were hints to the contrary in the literature. William Harvey had said, "and therefore the yolke seems to be a remoter and more deferred entertainment than the white, for all the white is quite and clean spent before any notable invasion is made upon the yolke". Another important observation was that of Prévost & le Royer in 1825, who obtained from the allantoic fluid of a 17-day old chick a nitrogenous substance which gave an insoluble nitrate and resembled urea in all particulars. There had clearly been some catabolism of proteins. And since at about the same time Jacobson; Sacc; and Stas found uric acid in considerable amounts in the allantoic fluid of developing chicks during the

third week of incubation, there was no doubt about it. In 1925 I pointed out that, although fat was the predominating energy-source in the chick embryo, it could certainly not be the only one, for many arguments indicated carbohydrate and protein as the energy sources of the initial stages.

1. In the first 8 days of incubation there is a striking fall in the amount of free glucose. The curves of Satô; Idzumi; Bywaters; and Tomita, all show a rapid fall from about 0·4 gm. per cent. to 0·1 or less.

2. Simultaneously with this disappearance of free glucose, the lactic acid in the egg, which has previously been low, reaches a peak and immediately afterwards descends to its previous level (Tomita). From an initial level of 0·02 mgm. per cent. it attains on the 5th day a maximum of 0·13 mgm. per cent., and regains its original value about the 14th day. That it was intimately connected with glucose metabolism he proved by injecting glucose, and observing an increase of lactic acid.

3. When the figures of Bohr & Hasselbalch were examined (Fig. 144, p. 703), it was seen that, although the respiratory quotient during the last two weeks of incubation is 0·73, in the earlier stages it is nearly as high as unity[1].

4. The disappearance of glycogen in very early stages of development has been investigated by Lewis and Konopacki. The former grew tissue cultures of cells from very young chick embryos, and could never find glycogen present in them after the first 50 hours of development. Konopacki, working on the frog, obtained exactly similar results. He found that after fertilisation and the formation of the perivitelline space the glycogen greatly diminished in quantity and remained very low until the neurula stage was reached, after which the glycogen rose again. Similar results have recently been reported by Vastarini-Cresi. All these workers made use of histochemical methods.

5. We saw evidence pointing in the same direction in the work of Warburg, Posener & Negelein. They found a very marked preferential consumption of carbohydrate on the part of 3–5 day chicks' tissue. The production of ammonia when the tissue was suspended in bicarbonate Ringer *in vitro* was at once suppressed, if sugar was provided for it. Negelein also found that the power of glycolysis is very high in early development, and lower in the adult condition.

[1] And Dickens & Simer found quotients of unity for 5th day chick embryos *in vitro*.

6. Perhaps it is no coincidence that the respiratory quotient which is found in the early cleavage stages of small marine eggs such as those of the sea-urchin is in the near neighbourhood of unity. Warburg's respiratory quotient of 0·9 for the first 24 hours of the development of *Arbacia pustulosa* eggs and Shearer's respiratory quotient of 0·95 for *Echinus microtuberculatus* are relevant here. A higher figure still was found by Fauré-Fremiet, whose results with the eggs of *Sabellaria alveolata* worked out at a respiratory quotient of 1·0. As indices of the nature of the combustions going on these figures have to be accepted with caution, but it is natural to suppose that a stage which lasts 5 days in the chick may last only a few hours in lower animals which develop faster, so that the time to look for utilisation of carbohydrate might be from fertilisation to the gastrula stage.

7. Then Simon and Susanna Gage fed laying hens on Sudan III and found that, although red yolks were laid, the embryos showed no trace even of a pink colour until the 10th day of incubation. After that time they became rapidly more coloured, until at hatching they were quite red. One would suppose that, in order to be combusted, fat would have to be absorbed into the embryo, and would take the dye with it, as indeed did happen after the 10th day. Murray's estimations of fat storage in the embryonic body confirm this (see Fig. 362), and point to an awakening of fat metabolism after the mid-point of incubation. Then Tallarico and Remotti have brought forward evidence showing that the lipase of the yolk increases markedly in activity towards the end of incubation, while the latter worker finds a precise correspondence between proteolytic activity of yolk and intensity of protein combustion at the 8th day of development.

8. The work of Riddle on the yolk and the yolk-sac is interesting in this connection. From the 15th to the 20th day the yolk is practically the only supply of the chick, and his chemical study of it during this period showed that there is a preferential absorption of fat. This agrees exactly with the absorption curve. The neutral fat decreases markedly in the yolk and the protein substances increase.

9. The earliest statement that protein in the hen's egg must be a source of energy is due to Sznerovna. She found a constant relationship between the nitrogen in the embryo and the nitrogen in the allantoic fluid, as 17 to 1.

10. Bialascewicz & Mincóvna, working on the frog's egg, found that up to hatching there had been practically no loss of fat, but

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that a loss of protein had certainly taken place. Parnas & Krasinska found no loss of fat during this time. Fauré-Fremiet & Dragoiu also studied the frog's egg; they agreed with Bialascewicz & Mincóvna in finding a loss of protein before hatching, but they also observed a loss of glycogen and of fat, indicating that all three substances had acted as sources of energy.

11. The same conclusion was arrived at by Farkas for the egg of the silkworm, *Bombyx mori*.

12. Dakin & Dakin found a utilisation of proteins during the development of the eggs of the plaice, and Greene observed the same thing in the king-salmon.

13. If urea and uric acid are indicators of protein metabolism, so also is the phenomenon of specific dynamic action. We have already seen evidence that there is a period in the development of the chick when this phenomenon appears (p. 935). Gayda's work on the heat given out by toad embryos throughout their development, showed that the heat eliminated during periods in which the weight was doubled, plotted against the time, gave a curve with a peak in the centre, to which the values rose and from which they descended. Thus development was more economical at the beginning and end of development than at the middle, just as in the chick.

14. Pigorini's work on the embryos of *Bombyx mori*, in which the glycogen was estimated throughout their development, fell into line with the other researches on carbohydrate utilisation mentioned above, and confirmed the older work of Vaney & Conte. The last-named investigators found that not only glycogen but also fat fell during the development of the silkworm.

15. The only work which has been done on the chemical constitution of the echinoderm egg during its development fits in well with the high respiratory quotients found for the beginning. Ephrussi & Rapkine found a fall in protein, fat and carbohydrate during its development.

16. In the chapter on respiration and heat production, attention was drawn to the fact that Meyerhof's calorific quotients, obtained during the early development of the echinoderm egg, did not correspond with the theoretical values, either for carbohydrate, protein, or fat combustion. Shearer's later values, which were based on a rather greater heat production, came nearer, and finally Rogers & Cole, finding still more heat, gave data from which calorific quotients

very near the carbohydrate level can be calculated. This was first pointed out by Needham in 1927, who suggested that these researches could be interpreted as a progressive elimination of leaks.

17. Indirect evidence about the utilisation of protein by the embryo can be gained from the work of Scheminzki, who determined the resistance of the trout egg to damage by electric currents during its development. The whole period was 55 days, and for the first 30 days there was practically no change in the resistance, but after that time it rose tremendously, the strength of current required to produce precipitation of the ichthulin in one minute increasing six times in the last 25 days of development. The effect of the current was to render the egg-membrane permeable to cations, which diffuse out and cause the ichthulin to be precipitated. Jarisch showed that lipoids and fats in systems poor in salt favour the precipitation of globulin, so if the current dismisses the cations from the egg, the precipitation of ichthulin will be more favoured the more fatty substances there are present. Scheminzki's curve becomes, then, in some measure an index of the amount of fat absorbed by the embryo, and the fact that it is of so gradual a slope during the first two-thirds of development may be interpreted as showing a greater intensity of fat absorption (and combustion?) towards the end of development than towards the beginning. These findings may be compared with those of Gage & Gage on the chick embryo.

18. Besides Tomita and Gräfe, a few other investigators drew attention in the past to evidence showing that fat was not the only energy source of the chick embryo. Dröge considered that protein must take a share in the work, and Sakuragi specifically went into the question of the other energy sources of the embryo. In the German summary to his Japanese paper, he says, "Obwohl bisherige Autoren, welche sich mit Stoff- und Energiewechsel von bebrütenden Hühnereiern beschäftigen, die Bedeutung des Kohlehydrates für Energiewechsel ganz vernachlässigten, glaubt der Verfasser, dass der schon vorhandene Traubenzucker in den ersten Bebrütungstadien besondere Wichtigkeit und grosse Bedeutung dafür hat, und dass der erste chemische Vorgang in den bebrütenden Eiern in der Zersetzung von Traubenzucker besteht". Sakuragi estimated the free and combined sugar, the fat, the various fractions of nitrogen, and the glycogen at the different stages of development, and interpreted his figures as showing that throughout development carbohydrate was

combusted, the fat at the late stages being turned into carbohydrate before being burnt. His arguments for this process, however, were not convincing.

19. A very striking support for the view that a succession of energy sources takes place in the development of the chick is to be found in the analyses of the white yolk which were carried out by Riddle and by Spohn & Riddle. As has already been mentioned (see p. 286), the white yolk, the earliest pabulum of the embryo, is much richer in salts and in protein than the yellow yolk, which forms its later nutriment. These facts fit in exactly with what has already been said about the constitution of the embryo and its sources of energy. It is almost safe to predict that the white yolk will be found to have a higher percentage of total carbohydrate, or perhaps of free sugar, than the yellow yolk.

20. When the ammonia, urea and uric acid in the hen's egg are estimated throughout incubation, the absolute amounts rise in regular curves corresponding to the growth of the embryo. When further analysis is made, however, it is found that in all cases the amount of these nitrogenous end-products, when related to wet weight, rise up to a certain point, and then remain at a steady level, while, if they are related to dry weight, they rise to a peak from which they fall again in the later stages of development. Thus in each case it could be said that 1 gm. dry weight of embryo excretes a maximum of nitrogenous waste at a definite point in development. The obvious conclusion is that a peak of maximum protein catabolism exists, coming significantly at 8·5 days of development, i.e. exactly between the periods which, on the evidence given above, we believe to be associated with the predominant catabolism of carbohydrate and fat respectively. Fig. 261 shows these relationships, plotting the protein combusted in milligrams per cent. of the dry or wet weight of the embryo at the time against the age. In Fig. 262 the curve of Fiske & Boyden is also given. The investigations of Bialascewicz & Mincóvna have also made it likely that a similar peak exists in the frog embryo. Fig. 368, constructed from their data, illustrates this strikingly, for in it the quantity of nitrogen in milligrams excreted by one embryo in each 24 hours is plotted against the age in hours from fertilisation. An unmistakable peaked curve is seen, and confirmation of the views already expressed is furnished by the curve for combustion of fatty acids, which rises steadily but *later* than the nitrogen excretion curves.

The work of Bialascewicz and Mincóvna will be considered in more detail in Sections 9·9 and 11·3.

The conception of an ontogenetic succcession of energy sources has to reckon, however, with a few facts which do not easily fit it. Perhaps the most difficult phenomenon to explain from this point of view is the apparent combustion of carbohydrate exclusively by mammalian embryos (Bohr). It must be admitted that the evidence for this is slender, but even if it were true, it would not be surprising, as most living cells combust carbohydrate if they can get it, and the continuous perfusion system of viviparity may provide such a supply. Perhaps mammalian embryos might be regarded as having prolonged their carbohydrate period to cover the whole of their pre-natal life, and, if this were so, the peaks in basal metabolic rate found by Wood; DuBois, and others on mammals shortly after birth might be associated with maximum intensities of protein combustion.

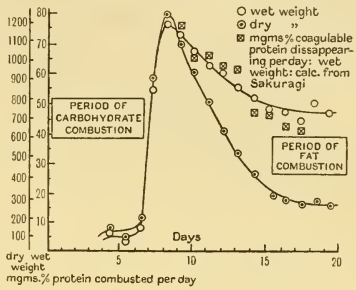



Fig. 261.

Probably other substances besides protein, fat and carbohydrates may be utilised to supply energy in some forms of life. For example, the recent discovery by Heilbron of great amounts of spinacene, a cholesterol-like substance, in selachian eggs, may lead to the solution of the problem of the energy-source of these eggs. What they combust has so far been quite unknown.

Gräfe in 1910 thought that there might be some connection between the period of carbohydrate utilisation in the chick's development and the fact that at that time the most profound morphogenetic changes were going on. And it has been suggested that the fat period at the end might be associated with preponderance of change of size over change of shape. But perhaps the time has not yet arrived for correlations of this kind. Again, some connection may appear between the succession of energy-sources in ontogenesis and the numerous observations of susceptible stages in development, such as those of

Stockard and of Parnas & Krasinska. This work has brought out with exceptional clearness the fact that development may be discontinuous, and in all cases passes through critical stages when disastrous effects will follow an interference innocuous at other times. The beginning of gastrulation is such a critical stage. Stockard says, "The present

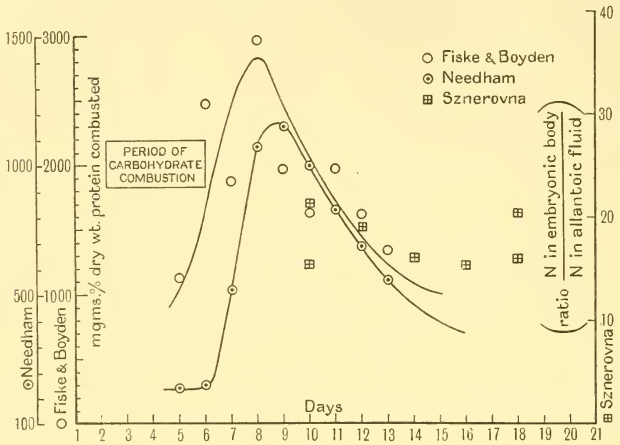



Fig. 262.

extremely crude state of our knowledge of the chemistry of development will permit of no...satisfactory statement of the principles underlying differences in developmental rate". Critical stages in development may turn out to be associated with changes from one type of substance to another type as a source of energy. An intermediate link in the chain of events would be the rapid growth-rate of one or more organs, leading to a teratological result if development was at that moment interfered with.

The ultimate nature of the succession of energy sources presents a problem of some interest. It is possible that carbohydrate is first combusted because it requires no preparation. Proteins must be deaminated, fats must be desaturated, and probably the embryo in its earlier stages cannot do either of these things, but, on the other hand, glucose lies ready for use, and it is significant that what is then

combusted is free, not combined, carbohydrate[1]. There is already evidence that the power of desaturation of fats only arises at a comparatively late stage of development, e.g. the 10th to the 15th day in the chick (see p. 1171). And we may look on the unsaturated fatty acids which are notably present in the yolk (see p. 295) as a preparation for these conditions.

Or it may be that some conception of "ease of combustion" will prove helpful. Quastel & Whetham, studying the action of *B. coli* on various organic substances, found that carbohydrates were much better hydrogen donators than substances of the protein or fatty type. The following figures, taken from their paper, are striking.

| Substance | | Reduction coefficient (The reciprocal of the molar concentration required to reduce 1 c.c. of 1/5000 methylene blue in presence of a standard amount of organism in half an hour) |
|---|---|---|
| "Carbohydrate". | Glucose ... | 5000 |
| | Fructose ... | 5000 |
| | Lactic acid ... | 583 |
| "Protein". | Alanine ... ... | 1 |
| | Glycine ... ... | 0·8 |
| | Glutaminic acid ... | 25 |
| "Fat". | Nonylic acid ... ... | 0·4 |
| | Heptylic acid ... ... | 0·4 |

The succession of energy sources might, then, be related in some way to the changing *r*H of the embryonic cells or to other important factors in the oxidation-reduction processes going on in them. A study of the oxidation-reduction properties of embryonic cells throughout development would be interesting. Aubel & Wurmser have made some progress in this direction.

The ontogenetic succession could be either "ovogenic" or "embryogenic". On the former view, the energy sources would succeed one another simply because the dynamic equilibria and the relative concentrations of substances in the yolk and white necessitated it. The embryo would play a passive part, combusting protein and fat only since it could not get carbohydrate. On the latter view, the succession of energy sources would be intimately connected with the changing potentialities of the growing embryo. Energy must be the same from whatever source it comes but the embryo—on this view

[1] And yet even here a preliminary combination with phosphoric acid may be necessary.

Table 126. *Energy sources*

| Animal | Amount of substance present at beginning (mgm.) | | | Amount of substance present at end (mgm.) | | |
|---|---|---|---|---|---|---|
| | Carbohydrate | Protein | Fat | Carbohydrate | Protein | Fat |
| ck (*Gallus domesticus*) ... ... ... | 335 | 6375 | 5600 | 170 | 4890 | 3450 |
| g (*Rana temporaria*) ... ... ... | 0·040 | 1·14 | 2·55 | 0·037 | 0·84 | 1·59 |
| ok trout (*Salvelinus fontinalis*) ... ... | — | 13·7 | 6·4 | — | 10·6 | 6·7 |
| nt Salamander (*Cryptobranchus allegheniensis*) | — | 40·25 | 11·18 | — | 38·28 | 12·74 |
| ke (*Tropidonotus natrix*) ... ... ... | Losses not known but combusted material considered to be (of at | | | | | |
| mon ... ... ... ... ... | — | 26·8 | 10·1 | — | 13·0 | 4·2 |
| worm (*Bombyx mori*) ... ... ... | 1·98 | 11·31 | 8·08 | 0·74 | 9·2 | 4·37 |
| ice (*Pleuronectes platessa*) ... ... ... | — | 0·213 | 0·0057 | — | 0·174 | 0·0204 |

* 0·033 calculated

playing an active part—would combust such and such substances at such and such periods of its development because it would not have at those times the capacity for combusting others. The molecular orientations on its intracellular surfaces would differ at different stages of its development, and its enzyme systems would vary profoundly in activity.

At present there is not enough evidence to allow us to make a final choice between these views. The ovogenic hypothesis would commit us to the belief that, if sufficient carbohydrate were present during the protein and fat combustion periods, the utilisation of these latter by the embryo would greatly diminish or disappear. On the embryogenic hypothesis we should have to believe that, however much carbohydrate were present during the protein period, the embryo would continue to combust protein, for a close relationship would exist between its source of energy and its stage of development. In favour of the ovogenic hypothesis might be cited the case of the viviparous embryo, which may possibly combust carbohydrate throughout its development. But dangers beset any direct comparison between embryos *in ovo* and *in utero*. Mammalian embryos have a continuous perfusion system, non-mammalian embryos have not; so that in one case the proportion of embryo to nutriment does not alter, and in the other case it does. Mammalian embryos can have all their combustible material supplied to them in solution; if the avian embryo

*of various developing embryos.*

| Substance lost, i.e. combusted (mgm.) | | | | Substance combusted in °/₀ of total material burnt | | | Substance combusted in °/₀ of substance originally present | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Carbohydrate | Protein | Fat | Total | Carbohydrate | Protein | Fat | Carbohydrate | Protein | Fat | Investigator |
| 20 | 69 | 2150 | 2250 | 3·02 | 5·57 | 91·4 | 10·0 | 7·5 | 38·8 | Murray; Needha |
| 0·029 | 0·300 | 0·095 | 0·424 | 6·84 | 70·70 | 22·4 | 7·2 | 26·0 | 4·0 | Barthélemy & Bo net; Needham |
| — | 3·1 | ? | 4·8 | — | 63 | 37 | — | 21·96 | ? | Gortner; Tangl Farkas |
| — | 1·97 | ? | 0·97 | — | 100? | ? | — | 4·9 | ? | Gortner; McCle don |
| principally carbohydrate by Bohr, who obtained a regularly high respiratory quotient | | | | | | | | | | Sommer & Wetz Bohr |
| — | 13·8 | 5·9 | ? | — | 60·0 | 30·0 | — | 55·0 | 60·0 | Greene; Miesche |
| 1·24 | 2·11 | 3·71 | 5·31 | ? | <10·0 | 64·0 | — | 18·7 | 46·0 | Tichomirov; Kel |
| — | 0·039* | ? | 0·031 | — | 100·0 | — | — | 15·0 | — | Dakin & Dakin |

least 0·90)

from oxygen consumption.

lived in the same style it would need an egg vastly larger than its present size to contain its physiological sugar solution. The fat of the avian egg is tabloid food.

The active autohegemonic powers of growth which the embryo has been shown to possess by the experimental embryologists might seem to favour the embryogenic hypothesis. In its support could also be adduced the fact that, during the protein period in the avian egg, there is plenty of carbohydrate being absorbed in the bound form of ovomucoid. It will be seen in Section 8·2 that the free sugar—probably the only carbohydrate fraction burnt by the embryo —does not entirely disappear until the 12th day of development. Yet, as Fig. 261 shows clearly, it is between the 8th and the 9th day that the peak of utilisation of protein occurs.[1] The embryo then by no means awaits the exhaustion of its carbohydrate supplies before beginning to combust protein. This fact is strong evidence in favour of the view that the embryo and not the supply of food at its disposal is in command of the situation. In order, however, to make more certain of this, I carried out some injection experiments. Eggs were injected with a solution of glucose containing 500 mgm. per c.c. By injecting into the air-space the mortality of embryos is much reduced. As will be seen from Fig. 263, no significant effect was produced upon

[1] Moreover, at that moment the egg also contains about 140 mgm. per cent. of glucose in the bound form of ovomucoid.

the uric acid curve. If the embryo had been burning protein because carbohydrate was absent or not easily obtained, then the uric acid curve should have been depressed after the injection of glucose, but this was never the case.

So far the order in which the three great types of biologically important molecule are combusted for the Ea. during the development of the embryo has alone been taken into consideration, and not the relative proportions in which they are used for this purpose. In the case of the chick, the following figures were obtained by Needham in 1927:

| | Total material disappearing during the whole of incubation (mgm.) | Correction for material disappearing but not combusted (mgm.) | Result | Same in per cent. of the total material catabolised |
|---|---|---|---|---|
| Carbohydrate... | 166 | 40 | 126 | 5·57 |
| Protein ... | 68 | 0 | 68 | 3·02 |
| Fat ... ... | 2171 | 105 | 2066 | 91·4 |
| | | | 2260 | |

This is simply a more accurate statement of a fact which has frequently been mentioned before, namely, that the main source of Ea. in the hen's egg is fat. Other embryos, however, do not utilise fat to the same extent, and Table 126 summarises the data which show this. It largely explains itself. The first two items are the only ones in which our knowledge is complete, though doubtless not final. Attention may be directed to the fact that the amount of carbohydrate combusted in per cent. of the total amount of food-stuff combusted is of the same order in the frog as in the chick. But this is not the case for protein and for fat, for in the former case 71 per cent. is protein and 22 per cent. fat, while in the latter case 6 per cent. is protein and 91 per cent. fat. Here then is a distinct difference between the metabolism of the embryonic chick and the embryonic frog. The problem is to decide upon what biological difference to fix as the correlate of this biochemical difference. Most probably the

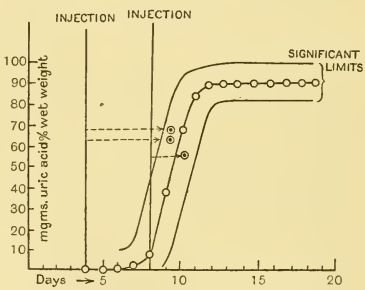


Fig. 263.

difference is associated with the difference between terrestrial and aquatic forms. Generally speaking, aquatic embryos use a great deal of protein during their ontogeny, but terrestrial ones are sparing of it.

This generalisation may, at any rate, be regarded as a legitimate working hypothesis, and fits in with Gray's distinction between aquatic and terrestrial embryos, in that the former can obtain water for their tissues from their environment, while the latter cannot, so that an apparatus has to be provided for storing the necessary water in the egg, and supplying it at a steady rate to the embryo. The egg of the trout, for instance, contains, like that of the chick, enough solid to make one embryo, but, unlike that of the chick, not nearly enough water. In the same way, the embryo of the trout, on the view here propounded, need exercise no economy in the combustion of protein, since it has an unlimited space into which to excrete the resulting waste products, but the embryo of the chick, on the other hand, has only a very limited means of disposing of such compounds, and accordingly obtains its Ea. from substances that burn away completely to carbon dioxide and water. This subject will be more fully discussed in Section 9·15.

## SUMMARY OF DEFINITIONS

Ea (Tangl) = U (Rubner) = the "Entwicklungsarbeit," i.e. the chemical energy in that fraction of the raw materials of the egg, which is combusted by the embryo during its development.
REa (Tangl) = the Relative Ea, i.e. the Ea calculated for 1 gm. wet weight of embryo.
SEa (Tangl) = $U_I$ (Rubner) = the Specific Ea, i.e. the Ea calculated for 1 gm. dry weight of embryo (Tangl) or 1 kilo dry weight (Rubner).
OE = "Organisation-Energy," i.e. the energy stored in the embryo which, though appearing as calorific value of combusted wet tissues, would not result from the combustion of an unorganised mixture of its constituent chemical substances.
Wa (Tangl) = "Wachstumsarbeit," i.e. that fraction of the Ea which is due to the function of Growth.
Na (Tangl) = "Neubildungsarbeit," i.e. that fraction of the Ea which is due to the function of Differentiation.
Ua (Tangl) = "Umbildungsarbeit, i.e. that fraction of the Ea of insect metamorphosis which is due to the functions of Transformation and Rearrangement.
Ha = "Histolysearbeit," i.e. that fraction of the Ea of insect metamorphosis which is due to the function of Histolysis.
$U$ (Terroine) = the amount of chemical energy stored in the raw materials of the egg.
$U_E$ (Terroine) = the amount of chemical energy in the unused raw materials at the end of development.
$U'$ (Terroine) = the amount of chemical energy stored in the finished embryo.
W (Rubner) = $U'$ calculated for 1 kilo wet weight of embryo.
$W_I$ (Rubner) = $U'$ calculated for 1 kilo dry weight of embryo.
$U_E$ (Terroine) = that fraction of the Ea which is due to basal metabolism.
AEE = Apparent Energetic Efficiency, i.e. the relation between the chemical energy in the material stored, and that in the material combusted during development, ($U'$/Ea).
REE = Real Energetic Efficiency, i.e. the relation between the chemical energy in the material stored and that in the material combusted during development for non-basal metabolism only ($U'$/Ea − $U_E$).
SEE = Synthetic Energetic Efficiency, i.e. the relation between the chemical energy furnished to the embryo by coupled reactions with one endothermic component, and that in the material combusted during development for non-basal metabolism only.
PEC = Plastic Efficiency Coefficient, i.e. the relation between the material (*not* the energy) stored in the embryo, and the material combusted by the embryo.

## SECTION 8

## CARBOHYDRATE METABOLISM

### 8·1. General Observations on the Avian Egg

Our knowledge of the carbohydrate substances in the egg of the hen begins, where so much embryological history begins, with William Harvey, who in the *De Generatione Animalium* says, "Egges after two or three daies incubation are even then sweeter relished than stale ones are. And after full fourteen daies (when the Chicken now beginneth to be downey and extendeth his Dominion over halfe the egge and the yolke is almost still entire) I have boyled an egge till it was hard that so I might discerne the position of the chick more distinctly—and yet the yolke was as sweet and pleasant as that of a new laid Egge when it is likewise boyled to an induration". Compare with this, Fig. 275.

The subject of the carbohydrate metabolism of embryonic life is not a difficult one to deal with if a few initial propositions are kept in mind. Thus, as will appear later, glycogen cannot be considered as a representative carbohydrate, although some workers have assumed that it is. Then the earlier work has all to be judged in the light of the fact that copper reducing methods, so universally used for determining glucose quantitatively, give results which are far too high in the presence of protein breakdown products. The earlier work can accordingly be used only when there is reason to believe that peptides and amino-acids were excluded, as in the determination of free glucose after dialysis or precipitation of proteins, and not when the estimations were carried out on protein hydrolysates. Holden found that the Hagedorn-Jensen method, which involves the reduction of ferricyanide not of copper, was the most reliable, and the subsequent work of Pucher & Finch; Duggan & Scott; Fazekas; and Jonsell, Jörpes & Sikström has demonstrated the same thing. Passing now from questions of technique, it will be best to use our knowledge of the carbohydrate metabolism of the hen's egg as the skeleton on which to build up this chapter, for it is by far the best known case, and possesses all the important features, of embryonic carbohydrate metabolism.

## 8·2. Total Carbohydrate, Free Glucose and Glycogen

The first question which presents itself is naturally what happens to the total carbohydrate in the hen's egg during development, and how it is transferred to the embryo. The impossibility of estimating glucose by copper reduction methods in the presence of amino-acids as in protein hydrolysates makes the figures of Sakuragi for total carbohydrate uncertain, and the only available ones are those of Needham, who used the Hagedorn-Jensen method, after precipitating the hydrolysate with phosphotungstic acid. Hydrolysis was carried out with 5 per cent. hydrochloric acid for 5 hours. In this way the amount of total carbohydrate, i.e. free carbohydrate plus carbohydrate combined with proteins plus glycogen, was determined each day during development (*a*) for the embryo and (*b*) for the rest of the egg. Fig. 264 shows the former values; they rise as the embryo grows very regularly from 1/10th mgm. on the 3rd day to 70 mgm. at hatching. Similarly, Fig. 265 shows the behaviour of the total carbohydrate outside the embryo; it first of all falls, then rises again somewhat, after which it falls again till the end of incubation. Evidently by adding the data of Fig. 264 and Fig. 265 together, we obtain the amount of total carbohydrate in the whole egg on each day of development. This is shown in Fig. 266, from which it appears

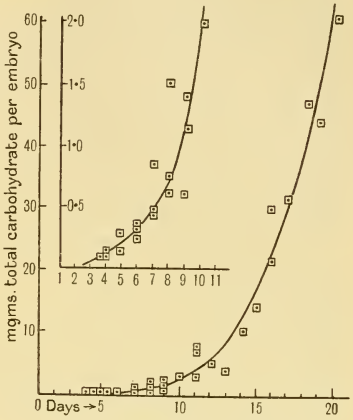



Fig. 264. The inset shows the early portion of the curve enlarged.

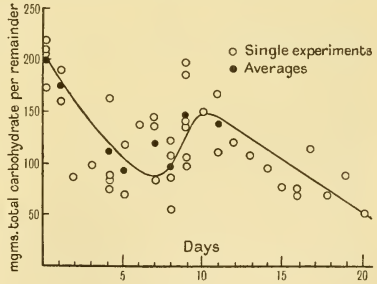



Fig. 265.

that at first the total carbohydrate in the whole egg falls, then rises again a little, and afterwards maintains a more or less horizontal course till hatching. In other words, the loss from the yolk and white after the 10th day is practically compensated for by the gain of the embryo, but in the earlier stages this is not the case. Beside the main data on Fig. 266 are placed a few other points, some obtained by Sakuragi, using his own method and some which I obtained using that of Wood & Ost; both these depend on the reduction of copper, and gave higher results than those of the Hagedorn-Jensen method, but they also show a constancy of total carbohydrate in the latter half of incubation.

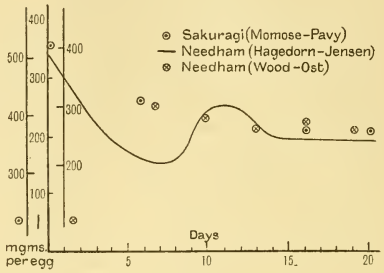


Fig. 266.

Having ascertained the movements of the total glucose during the chick's development in its egg, we may proceed to consider the movements of the various fractions into which it is divided.

It will first of all be of interest to compare the total carbohydrate of the remainder with the uncombined glucose there during the first half of development. Figures for the free sugar exist in some number in the literature, and, although all are derived from experiments in which copper reduction methods were used, they are yet worthy of credence, because total hydrolysis with its production of amino-acids was not involved. Creatinine and glutathione would probably not be present in the protein-free filtrates, so that the objections against copper methods discussed above are not grave in the case of free sugar.

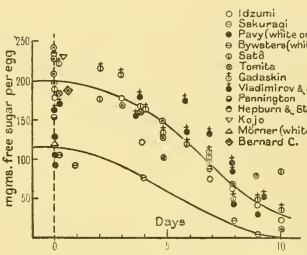


Fig. 267.

In Fig. 267 the figures of the various observers for the free glucose are summarised together. The sets of data are twelve in number,

namely, those of Idzumi (Momose-Pavy method), Sakuragi (Momose-Pavy method), Pavy (Pavy method), Bywaters (Pavy method), Satô (Schenck-Bertrand method), Tomita (Schenck-Bertrand method), Gadaskina (Galwialo method), Vladimirov & Schmidtt (Hagedorn-Jensen method), Pennington (method unknown), Hepburn & St John (Folin-Wu method), Kojo (Fehling method) and Mörner (Fehling method)[1]. In addition, the figures of Claude Bernard & Dastre, the first of all, dating from 1879, are included. It will be admitted that Bernard's values are remarkably accurate in spite of the crude methods at his disposal. It is striking that with diverse methods the results are in such good agreement, and a glance at Fig. 267 convincingly shows that the free glucose in the yolk and white diminishes considerably during the first half of incubation.

In some cases investigators only give their results in terms of percentages. In order to reduce them to a common basis, therefore, it has to be assumed that they all worked with normal eggs under approximately the same conditions. From the data given in Table 1, it may be assumed that of the weight of the entire egg at zero hour of development, 10·47 per cent. is accounted for by the shell, 56·07 per cent. by the albumen, and 33·46 per cent. by the yolk. Using Murray's figure for the weight of an egg (average of over 500), namely, 57·8 gm., the white will weigh 32·04 gm. and the yolk 19·33 gm. The change in weight during early development due to loss of water by evaporation, assuming a constant humidity, can be read off on the graph given by Murray. The varying water-content of yolk and white due to the current of water yolkwards can be obtained from Fig. 225.

The free glucose beginning at a maximum of 200 mgm. per egg sinks more or less steadily till the 10th day. An interesting point is the difference between the yolk and the white. Pavy and Bywaters only estimated the sugar in the albumen, and their points give a curve on a lower level than the whole egg curve, but roughly parallel with it. This might be taken to mean that there is not only a current of water but also a current of free glucose yolkwards, for by the 9th day the albumen has apparently lost all its free sugar, but the yolk has then lost only half its original amount. But Fig. 267 is misleading in that the values for the albumen are expressed as milligrams per egg, although the albumen does not alone account for anything

[1] See also the confirmatory data of Sagara (Schenck-Bertrand method).

like the entire egg. Fig. 268, in which the glucose is shown expressed as milligrams per cent. of yolk and white and plotted against time, is perhaps a better indication of what is going on. It demonstrates that, although both fractions of sugar fall markedly, there is a sort of cross-over in the middle of development, the yolk continuing at 75 mgm. per cent. and the white dwindling away to nothing. If a passage of glucose into the yolk takes place, it must be by way of

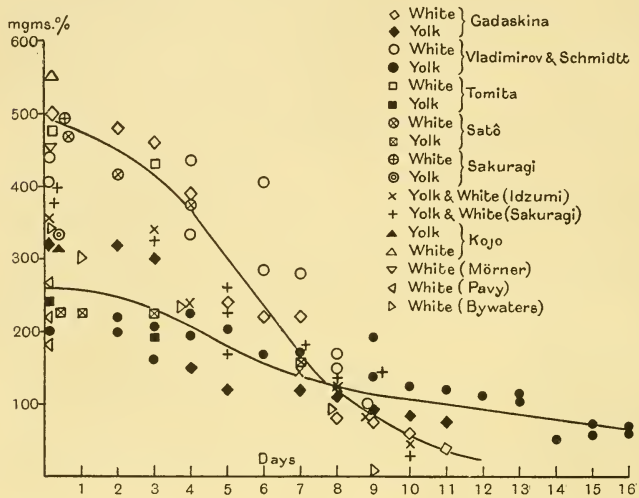



Fig. 268.

the chick's blood-vessels. There is a piece of experimental evidence against it, for in 1921 Tomita injected glucose into the air-space of unincubated eggs. The idea had previously occurred to Pouchet & Beauregard, who, as far back as 1877, had injected $\frac{1}{2}$ gm. of sterile "crystallised cane-sugar" into hen's eggs. Development was normal up to 13 days, but there was an odour of lactic fermentation. "Nous n'avons pu constater", they said, "la présence du sucre interverti dans l'albumine. Était-il demeuré dans son état ou avait-il disparu soit dans le vitellus, soit consommé par l'embryon?" Tomita's researches were more enlightening:

Table 127.

| Amount of glucose injected into the air-space before incubation | | Glucose found in the egg on the 3rd day of incubation (mgm. %) | |
|---|---|---|---|
| Mgm. of glucose | Mgm. % of egg-white | White | Yolk |
| 0 | 0 | 430 | 200 |
| 100 | 390 | 690 | 200 |
| 50 | 200 | 510 | 190 |
| 200 | 800 | 1080 | 190 |

| Amount of alanine injected into the air-space before incubation | | Glucose found in the egg on the 3rd day of incubation (mgm. %) | |
|---|---|---|---|
| Mgm. of alanine | Mgm. % of egg-white | White | Yolk |
| 100 | — | 410 | 180 |
| 50 | — | 400 | 190 |

Tomita proved, in short, that the amount of glucose in the white can be raised on any one day by injecting a supply into it at the beginning of incubation, in spite of the factors which are leading to its disappearance, but that the amount of glucose in the yolk cannot be changed in this way. In passing, it may be noted that the addition of alanine to the egg-white before incubation had no effect on the glucose-content of white or yolk. It would therefore seem improbable that glucose normally passes into the yolk from the white.

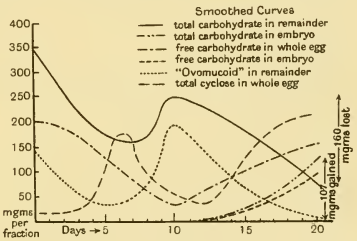



Fig. 269.

We can now relate the curve for disappearance of free carbohydrate with the curves for other carbohydrate fractions. In Fig. 269 it is shown in relation with the curve for total carbohydrate of the non-embryonic part of the egg. The total carbohydrate of the remainder falls from zero hour till the 8th day, rises from then till the 11th day, and thereafter falls steadily till the end of development. The free glucose also falls till the 10th day, but not quite in the same manner as the total carbohydrate, for at the beginning its fall is slow, and thereafter rapid, while the total glucose first falls quickly, and slows down as time goes on. The latter part of the curve for free glucose, as shown

in Fig. 269, is taken from the averaged estimations of Idzumi and Sakuragi, whose values closely agree.

Before discussing Fig. 269 further, the glycogen in the whole egg and in the embryo must be considered. Glycogen has been estimated in the embryo by Idzumi and Murray, and their figures agree well[1]. They are shown in Fig. 270. From these data it is simple to calculate the non-glycogen sugar in the embryo, and this is shown in Fig. 271 in its relation to the free sugar in the whole egg. It is seen that the rise in free sugar in the whole egg at the end of incubation goes almost exactly parallel with the rise of non-glycogen sugar in the embryo, maintaining a distance of about 60 mgm. from it. Thus at hatching there are about 50 mgm. of free sugar still remaining unabsorbed in the yolk-sac and presumably to be absorbed in the first few days of post-natal life.

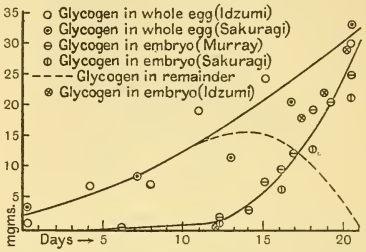



Fig. 270.

Since the free sugar in the embryo (or, more properly, the non-glycogen sugar) rises parallel in the last 10 days with the free sugar in the egg as a whole, it is evident that the free sugar outside cannot be the source of the free sugar inside, or, if it is, it must be constantly replenished from some other kind of carbohydrate. During this time the total carbohydrate outside (see Fig. 265) is steadily falling, and loses indeed in the last 10 days 160 mgm., during which time the embryo gains a total of 110 mgm. The difference must be either burned or transformed into some other substance, possibly cyclose.

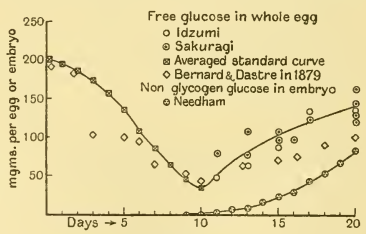



Fig. 271.

[1] Also subsequently by Vladimirov & Danilina, whose curve is almost exactly superimposable on that for the embryonic body in Fig. 270. Log. glycogen, they found, gives a straight line when plotted against log. age.

### 8·3. Ovomucoid and Combined Glucose

It is easy to calculate the amount of carbohydrate not present as glycogen or free glucose outside the embryo, for every day during development. This is graphically shown in Fig. 269. But such a calculation suffers from the fact that the non-glycogen sugar is assumed to be all free, which cannot be the case, but this error does not reach grave dimensions till the last 5 days of incubation, and as the correction would tend then to increase the free glucose outside the embryo it would tend also to decrease the ovomucoid glucose outside the embryo. We may therefore allow for the fact that the descent of this curve in Fig. 269 at the end of development is rather more precipitous than the graph makes it.

This curve, which, for want of a better name, we may call the "ovomucoid" curve, shows some interesting relationships. In the first place, its initial value, namely 133 mgm., is in fair agreement with the independent data of Komori, which have already been referred to (see p. 268). Komori prepared ovomucoid from fresh hen's eggs, obtaining from 333 gm. of albumen 4·8 gm. of ovomucoid. The processes were carried out as quantitatively as possible in order to get an idea of the concentration of the substance. This would mean 140 mgm. of ovomucoid glucose present at the beginning of development, which agrees very well with the 133 mgm. calculated from Needham's estimations by difference. This result gives us some confidence in interpreting the changes occurring in this fraction as changes in ovomucoid content.

What are these changes? As can be seen from Fig. 269, the ovomucoid curve falls until the 5th day is reached, after which point it rises to a peak on the 10th day, thence to fall steadily till the time of hatching. The initial fall is of great interest in view of Komori's experiments in which he showed that Müller & Masayama's egg "amylase" can very efficiently split off the sugar from ovomucoid. We may suppose that the heating of the egg at the beginning of incubation would set the enzyme in action, like the mechanism already suggested which controls yolk viscosity during the 1st week (see p. 836). By the 20th day there are at most half-a-dozen milligrams of ovomucoid left. All the carbohydrate outside the embryo at that time can be accounted for by glycogen and free glucose. From the fact that this peaked effect is found so markedly in the ovomucoid

curve the conclusion might be drawn that ovomucoid is a more labile element in the raw materials of the embryo than has usually been supposed. The work of Anson & Mirsky on another conjugated protein, haemoglobin, showed the ease with which the prosthetic group can be detached from and re-attached to the protein part of the molecule.

Komori gave other figures for the amounts of ovomucoid present during development, but expressed them as grams per cent. of dry weight of albumen, so that, although we know the rate at which the albumen is drying up, we cannot calculate his figures in milligrams absolute per egg because we do not know the relative weights of yolk and white. Sakuragi's figures for the same fraction are not valuable, being few in number and obtained by the use of doubtful precipitations prior to total hydrolysis and estimation by copper reduction. The only extensive work on the physiology of ovomucoid is that of Bywaters, who found that, between the 1st and 18th day of development, the ratio of uncoagulable protein nitrogen to coagulable protein nitrogen in the egg-white was steady at 0·136, and therefore concluded that there was no preferential absorption of ovomucoid or ovoalbumen. This does not at first sight agree with the curve shown in Fig. 269. Two hypotheses are open: (1) that the curve for ovomucoid glucose calculated by difference does not accurately represent the ovomucoid glucose, or (2) that at varying times in development the amount of glucose combined in the ovomucoid molecule varies considerably. Both these seem possible. Bywaters found no change in the amount of sugar in the uncoagulable protein between the 1st and the 18th day; it remained constant at about 27 per cent., and, as the ratio of the two expressed as grams per 100 gm. egg-white was more or less constant (see Fig. 272 and Table 128), he considered that the carbohydrate radicle of ovomucoid was not split off before absorption. His methods are, however, not free from criticism, for he used the original method of Pavy without modification, and only hydrolysed the ovo-

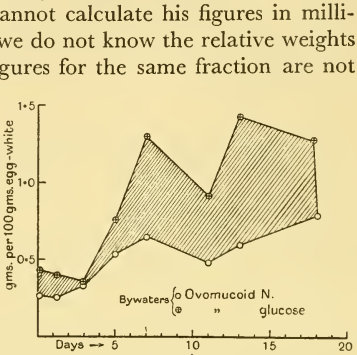



Fig. 272.

mucoid for 1·5 hours with 5 per cent. hydrochloric acid. Moreover his ratio varied from 1·0 to 2·4, which suggests that the hydrolysis was incomplete. It is significant that Bywaters' ovomucoid glucose values are highest between the 7th and 13th days, though their absolute values are at least 50 per cent. higher than mine. The second interpretation of the behaviour of the ovomucoid fraction receives some support from the work of Komori and of Levene & Rothen; for they have found the sugar to be combined with the protein as a polysaccharide. Further work on the constitution of ovomucoid and the changes which it undergoes during development is much needed.

Table 128. *Bywaters' figures.*

| Day | Ratio: Grams uncoagulable protein nitrogen per 100 gm. egg-white (wet)/grams coagulable protein nitrogen per 100 gm. egg-white (wet) | Ovomucoid in grams nitrogen per 100 gm. egg-white $\alpha$ | Ovomucoid glucose in grams glucose per 100 gm. egg-white $\beta$ | Ratio $\beta/\alpha$ |
|---|---|---|---|---|
| 0 | 0·17 | 0·27 | 0·42 | 1·6 |
| | 0·12 | — | — | — |
| 1 | 0·15 | 0·26 | 0·40 | 1·5 |
| 3 | 0·16 | 0·32 | 0·33 | 1·0 |
| | 0·12 | — | — | — |
| 5 | 0·16 | 0·53 | 0·76 | 1·4 |
| 6 | 0·14 | — | — | — |
| | 0·11 | — | — | — |
| 7 | 0·13 | 0·65 | 1·30 | 2·0 |
| 9 | 0·12 | — | — | — |
| 11 | 0·15 | 0·49 | 0·91 | 1·9 |
| | 0·10 | — | — | — |
| 13 | 0·13 | 0·60 | 1·43 | 2·4 |
| 16 | 0·11 | — | — | — |
| | 0·12 | — | — | — |
| 18 | 0·18 | 0·78 | 1·29 | 1·7 |
| | Average ... 0·136 | | | |

In 1927, I estimated the amount of ovomucoid in the egg-white during incubation, and also the percentage of glucose in the ovomucoid molecule. The percentage of the whole egg accounted for by the white descended from 21 to 0 per cent. The ovomucoid isolated in milligrams per egg fell from 128 to 4. Although a quantitative recovery of ovomucoid was not claimed (and indeed these figures are lower than those of Komori (see Fig. 273)), yet, as the same care was used throughout, it is probable that they have relative significance. When they are expressed as percentage of the wet weight of egg-white, it is interesting to note that the amount of ovomucoid

in 100 gm. of wet egg-white remains constant throughout development at about 1 gm. As far as these figures go, then, they confirm the observations of Bywaters, and indicate that there is no preferential absorption of ovomucoid from the white; although there may be from the yolk. This latter possibility is made likely by the fact that the hump on the ovomucoid-glucose curve comes just between the two times at which the mucoprotein-glucose inside the embryo is high.

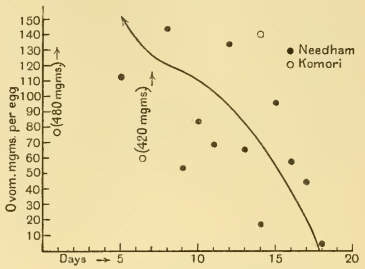


Fig. 273.

For the percentage of glucose in the ovomucoid molecule, the average value was 11·5, as has already been mentioned. This is probably more accurate than any other estimate, for it was obtained by the Hagedorn-Jensen method after phosphotungstic precipitation. But the curious thing about it is that, when the figures are plotted on a graph, as in Fig. 274, an upward trend is seen. No explanation has been found for this phenomenon. It is at any rate clear that there is no marked increase in the glucose content of ovomucoid at the period when it would be expected in the former of the two views outlined above.

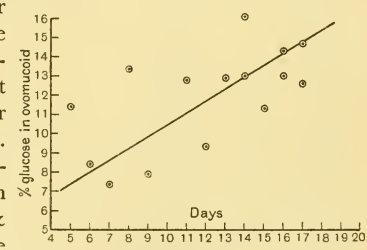


Fig. 274.

Continuing the subject of ovomucoid-glucose, we may return to the discovery by Müller & Masayama in 1899 of an active starch-hydrolysing enzyme in the yolk of unincubated hen's eggs. This amylase would convert "under favourable conditions" 45 per cent. of a 3 per cent. starch solution into the soluble forms of dextrin and isomaltose in 24 hours at 37°. This put on a sure basis the earlier and rather doubtful results of Krukenberg, and was in turn confirmed by Diamare; Herlitzka; and Roger. Idzumi brought forward data which showed that the activity of the extra-embryonic amylase markedly increased as development proceeded, especially after the 15th day (rising from 40

to 640 units). These researches will be referred to in more detail in Section 14·7. Finally Komori demonstrated that most, if not all, of the glucose in the ovomucoid molecule could be liberated by incubation with amylase prepared from meal.

It remained to demonstrate that the egg itself, or certain parts of it, possessed the power of hydrolysing ovomucoid, and I made the requisite experiments in 1927. The ovomucoid preparation itself contained no free glucose, but when incubated alone about 2 mgm. were split off, perhaps because of the presence of minimal amounts of the enzyme. This auto-digestion effect was subtracted from the crude results. It was noted in the first place that the slight excess of glucose in the embryo preparations after standing at 37° was much more than accounted for by the correction mentioned above; the effect of the egg-white was much reduced, but the yolk and the yolk-sac results still retained considerable magnitude. The enzyme ovomucoidase is thus not contained in the embryonic tissues themselves, and only to a negligible extent in the egg-white, but the yolk is very rich in it, and the blastoderm and yolk-sac also show a fair activity. Thus the embryo tests yield 0 per cent. of the theoretical, the white 1·49, the yolk 31·2 and the yolk-sac 7·13. This conclusion is not affected by the varying amounts of solid material which must have been contained in the original samples, for the yolk retains its superiority even when this is allowed for.

These results fit in well with the rest of our knowledge. Roger, for instance, found more amylolytic activity in the yolk than in the white. Bywaters' conclusion that ovomucoid was not split up into protein and sugar before absorption only holds for the white and for that fraction alone the results do not conflict. The ovomucoid may be pictured passing from the white to the yolk by the vitelline vessels, and there being split up into free glucose and protein before absorption into the embryo. It is interesting that the embryo possesses no ovomucoidase, or very little; the hydrolysis must therefore be considered to take place outside it. Idzumi's observations on the increase of activity of the yolk amylase during development also fit in with the increasingly rapid disappearance of ovomucoid seen in Fig. 269. It would be desirable to extend these observations by investigating the kinetics of the enzyme, and by determining whether its activity remains the same in all fractions during development.

The question now arises as to the value of the ovomucoid to the

embryo. It is probable that none of the ovomucoid is combusted during development, and the substance may therefore be considered of architectural rather than of energetical significance. This is in agreement with the main conclusion of Levene, who in his recent monograph ascribes to the mucoids in general a structural, cementing, and protecting value. From the ovomucoid curve in Fig. 269 we should expect to find some clues for its significance in the embryo about the 4th day and after the 11th day, because at both these times active ovomucoid catabolism is going on. And indeed it can be said that at these two points the needs of two tissues are at a relevant stage, namely, the primitive connective tissue and the bones. In the formation of these mucoprotein will be required, as if for the interstices of the growing embryo.

Von Szily first described a cell-free fibrous connective tissue ground-substance filling up all the cavities of the embryonic body in the early stages. This has some affinities with the "cardiac jelly" of Davis, and has recently been investigated by Baitsell, who has examined its properties with the aid of a micromanipulator. It appears to be secreted by the cells and provides them with a homogeneous matrix, a kind of natural culture medium, in which migration may take place if and when it may be necessary. As development proceeds the substance does not disappear, but becomes less and less important relatively to the body as a whole. The nearest equivalent to this ground-substance in later life is the Wharton's jelly of the umbilical cord and the vitreous humour of the eye. It is significant that both these tissues are known to be rich in substances of the mucoprotein type. It is probable that the decreasing importance of this primitive connective tissue may be related to the decreasing percentage of silicon in the embryo (see Section 13), and perhaps it may have some relation to the fall in the water-content of the embryo throughout incubation (see p. 871). The amount of glucose in the embryo each day not free and not present as glycogen can easily be ascertained. Its gradual rise follows the growth of the organism. But if we express it in percentages of weight of embryo, we find that 100 gm. dry weight of embryo contain 2550 mgm. of "mucoprotein glucose" on the 5th day, but only 980 on the 15th. This result is an interesting comment on the suggestion that the importance of the primitive intercellular matrix will require a high proportion of mucoprotein.

The fall of ovomucoid in the latter half of incubation may be

related to the process of bone formation, which, as von Baer established, proceeds at this time. Its calcium and phosphorus requirements have been studied in detail, and will be found discussed in Section 13·2, but very little attention has been paid to its need for osseomucoid. Even less is known about osseomucoid than about ovomucoid, but we may make a calculation which is suggestive. The amount of osseomucoid in fresh bone has not been accurately ascertained, and no estimation method has ever been devised for it. Hawk & Gies obtained 7 gm. of pure osseomucoid from 1700 gm. of fresh bone (0·04 per cent.), but they were not trying to work quantitatively. On the other hand, we know from the analyses of von Bibra; Schrödt; Wildt; Morgulis; and Weiske that the organic matter in bone amounts to about 33 per cent. of the fresh weight. The nearest approximation to the amount of osseomucoid would seem to be one-tenth of the organic substance. According to Tangl, the weight of the bones in the chick at hatching is 1·446 gm. dry weight, i.e. 3·616 gm. wet weight. This leads to an organic content of 1·205 gm. and about 120 mgm. of osseomucoid. Assuming its proportion of glucose to be 33 per cent., we get an equivalent in glucose of 40 mgm. Tangl's value for the weight of connective tissues at hatching is 0·405 gm. dry weight, or, assuming a water-content of 75 per cent., 1·615 gm. wet weight. The mucoprotein would here amount to about 60 mgm., and the corresponding glucose to 20 mgm. There would thus be 60 mgm. of mucoprotein-glucose present in the finished embryo. Since just under 200 mgm. of ovomucoid glucose have disappeared since the 10th day, it is evident that the mucoprotein of the raw material has some other goal besides the mucoprotein of the completed article, but the temporal correlation holds good, for, just as during the period of importance of connective tissue there was catabolism of ovomucoid, so here in the period of growth of bone there is a similar effect.

The closely related ovomucoid of reptile eggs has been investigated by Takahashi on those of *Thalassochelys corticata*. During development the percentage of ovomucoid in the organic matter of the egg-white varies from 33 to 17 per cent. The ovomucoid is attacked by amylase and yields 6·2 per cent. glucose (notably less than avian ovomucoid). Takahashi was inclined to think, on the basis of rather few analyses, that the elementary composition of the substance changed as the embryo developed.

Before leaving Fig. 269, attention may be drawn to the figures for inositol-content of the whole egg, as determined by Needham's method. Owing to the inferior accuracy of this method, which yet is the only available one, no stress can be laid on the actual values, but there is a reciprocal correlation between the total cyclose and the total carbohydrate.

## 8·4. Carbohydrate and Fat

So far the analyses which have been described were done in all cases upon the embryo and the yolk and white separately. Sakuragi's important paper of 1917 was based on analyses of the entire egg at different stages, and it is interesting to see how his results coincide with what has already been said. He adopted the plan of making a number of parallel observations with different methods, so that Fig. 275, which sums them up graphically, involves the following fractions:

A. (Hydrolysed residue after alcohol extraction) glucose of ovoalbumen, ovomucoid and glycogen.
B. (Alcohol extract after hydrolysis – D.), glucose of glycogen.
C. (Filtrate from protein coagulated by heat) free glucose.
D. (Alcohol extract before hydrolysis) free glucose.
E. (A. + B. + D.) total glucose.
F. (Hydrolysed residue after water extraction) glucose of proteins and of glycogen?
G. (Water extract) free glucose + ?
H. (Hydrolysed water extract with hydrochloric acid) free glucose + ?
I. (Total hydrolysis of whole egg with hydrochloric acid direct) total glucose.
J. (Ditto, a second time.)
K. (F. + H.) total glucose.

As Fig. 275 shows, Sakuragi did not observe any rise of total glucose in the egg as a whole between the 8th and 11th days. It is interesting that the free glucose determined in several different ways diminishes and then rises; this agrees with a great deal of earlier and later work. The glycogen, estimated directly, rises all the time. The unknown hydrolysable carbohydrate which is found in the watery extract has the effect of diminishing but not of completely abolishing the fall and subsequent rise of the free glucose. Its presence warns us that there may be many important processes in the carbohydrate changes in the developing hen's egg which are entirely hidden from us at present. The decrease in glucose combined with protein found by Sakuragi equates with the similar decrease in Fig. 269, though the course taken is very different, and Sakuragi himself made no attempt to explain it.

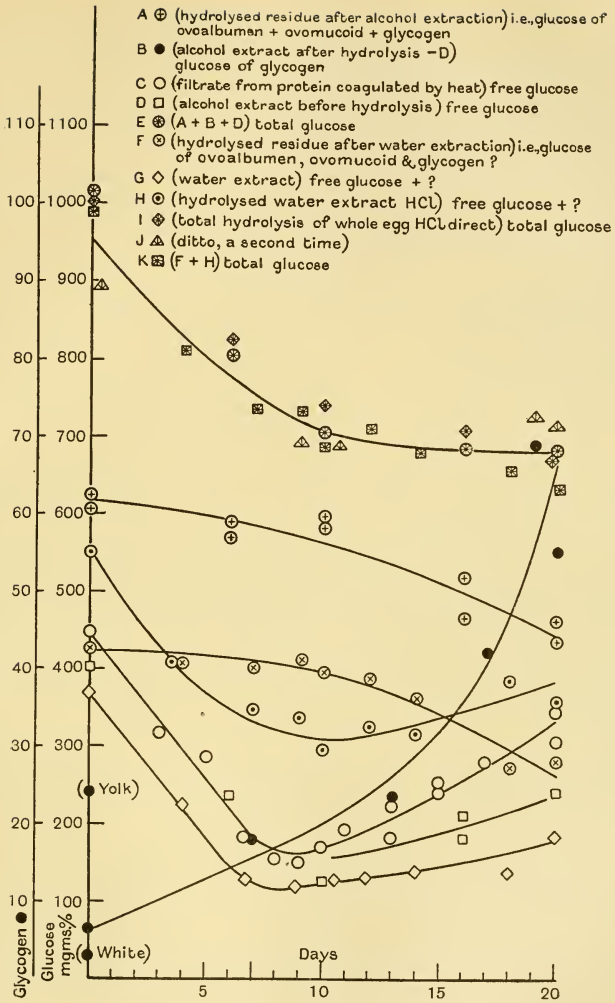

A ⊕ (hydrolysed residue after alcohol extraction) i.e., glucose of ovoalbumen + ovomucoid + glycogen
B ● (alcohol extract after hydrolysis −D) glucose of glycogen
C ○ (filtrate from protein coagulated by heat) free glucose
D □ (alcohol extract before hydrolysis) free glucose
E ⊛ (A + B + D) total glucose
F ⊗ (hydrolysed residue after water extraction) i.e., glucose of ovoalbumen, ovomucoid & glycogen ?
G ◇ (water extract) free glucose + ?
H ⊙ (hydrolysed water extract HCl) free glucose + ?
I ◈ (total hydrolysis of whole egg HCl direct) total glucose
J △ (ditto, a second time)
K ▩ (F + H) total glucose
110
100
90
80
70
60
50
40
30
20
10
1100
1000
900
800
700
600
500
400
300
200
100
(Yolk)
(White)
Glycogen ●
Glucose mgms%
Days
5
10
15
20

Fig. 275.

It is important to remember that he was dealing with the egg as a whole, so that any diminution in a substance or fraction means its absolute disappearance; as regards ovomucoid glucose, this strongly substantiates the conclusions drawn above[1].

Sakuragi considered that protein played no part as an energy source in the metabolism of the chick embryo. "It seems to be very interesting", he said, "that a small quantity of sugar is always kept undecomposed and moreover towards the end of foetal life some glycogen appears. The significance of this phenomenon may be interpreted as the necessity to convert the fat into sugar before it can be utilised as energy.... This is the reason why the egg contains a relatively high amount of sugar initially, sufficient to maintain the sugar balance until the organ capable of transferring fat into glucose has had time to develop."

However the kinematics of the inter-carbohydrate transformations may run, it is likely from Fig. 269 that the carbohydrate of the egg as a whole receives some reinforcement between the 8th and 11th days. This increase amounts to about 90 mgm., so protein is at once ruled out as a source, because during the whole of development only 68 mgm. are lost, and most of this must be due to true protein catabolism. It is true that the peak in protein catabolism occurs at just the same time as the gain of carbohydrate, but as between the 8th and 9th days the egg only loses 1 mgm. of protein while it gains 47 mgm. of carbohydrate this correlation is probably but a coincidence. We may safely conclude that the protein which is broken down is used for the production of energy, and being burnt away does not go to form that extra carbohydrate which appears in the middle of development.

The other possible source is fat, and here the position is a good deal more hopeful. As will be shown in Section 11·1, between the 7th and the 14th days there is a discrepancy between the fat lost as determined by the averaged chemical analyses and that determined by the carbon dioxide output on the supposition that all of it was due to fat, which is not true. More is lost than can be accounted for even on this assumption. "The figures of Bohr & Hasselbalch", I said in 1927, "would give an even worse divergence than those of Murray for they were lower than his. The explanation for this missing fat must be that during that period it is used for other

[1] See also Sagara.

purposes than combustion though there is, of course, the possibility that the estimations of fat are wrong." If now we suppose that the estimations were correct, and place beside the carbohydrate gained the fat missing each day, as is done in Fig. 276, we find that a correlation exists. Little account need be taken of the fact that the maximum of the carbohydrate gained is reached before the maximum of the fat lost, for the inferiority of methods, the comparative fewness of estimations, and the varying conditions of individual workers must all be borne in mind. Nor can stress be laid on the absolute magnitude of each quota, for an exact balance would show more carbohydrate gained than fat lost, since there is more carbon in a gram of fat than in a gram of carbohydrate. But it is striking that nowhere else in the development of the hen's egg can two such discrepancies be found, and that the two are approximately of the same order.

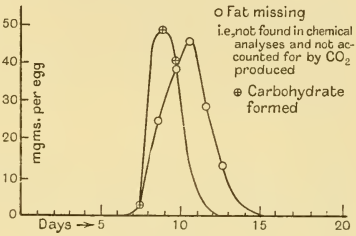


Fig. 276.

If then we accept the view that the increase in carbohydrate at this point is due to a transference from fat, there is one interesting corollary. It is that a transfer of a less oxygenated to a more oxygenated substance is taking place, and therefore that some oxygen would tend to be retained in the egg, and therefore that the respiratory quotient would tend to be lower than it theoretically should. If Fig. 144 be examined, it will be seen that there is a certain lowness of the experimentally determined respiratory quotient points as compared with those calculated from chemical analyses of food-stuff burnt. It is true that the passage from fat to carbohydrate, if it is of the order we are supposing, would not be large enough to make much difference, but it might well be one of several factors.

As is well known, the passage from fat to sugar is usually believed to be impossible *in vivo*, and it is certainly not supported by any unassailable piece of evidence drawn from experiments on the adult animal. In the starved dog the feeding of fat will not produce liver-glycogen, nor in the diabetic dog a rise in the G/N ratio, nor are any of the balance-sheet experiments, in which glucose seems to appear unaccountably and fat has to be postulated as its precursor,

free from criticism. In the present case, moreover, it is true that a passage through the form of cyclose might account for the fall and the rise in Fig. 269, but no stress can be laid on the high absolute values for the inositol in White Leghorn eggs, and further, if the carbohydrate goal is abandoned some other objective must be found for the missing fat. As evidence for the possibility of the fat-carbohydrate route *in vivo* these arguments give support to the experiments of Furusawa and of Calvocriado. Furusawa considered that the liver is the organ responsible for converting fat into carbohydrate, and, if this is so, it is interesting to note that the liver-cells of the developing chick can make carbohydrate from fat before they can store carbohydrate when it is made.

We must now examine this property of storing carbohydrate, and to do so the metabolism of glycogen in the embryo must be dealt with more fully.

## 8·5. The Metabolism of Glycogen and the Transitory Liver

The increase of glycogen in the embryo has already been mentioned. But we also possess figures for the glycogen in the whole egg owing to the investigations of Idzumi and Sakuragi, and, as they are quite concordant, confidence may be placed in them. They are depicted graphically in Fig. 270. By subtracting the glycogen in the embryo from the glycogen in the whole egg, we obtain that in the remainder, the figures for which appear as a dotted line in Fig. 270. By the end of development the glycogen in the embryo has only attained about a quarter of the total sugar in the embryo. It is interesting that Idzumi; Sakuragi; and Shaw all observed a great decrease of embryo glycogen during the process of hatching[1], Idzumi to 10 and Sakuragi to 25 mgm. per embryo. They consider that this is related to the vigorous muscular movements which the embryo then makes for the first time, including the lung movements, which come into play as the animal lays aside its allantois. As may be seen from Fig. 270, the glycogen is at its highest outside the embryo about the 13th day; after that time it rapidly falls, and is, indeed, more or less the reciprocal of the glycogen inside. Evidently after the 13th day the glycogenic function is shifted from somewhere outside the embryo to somewhere inside.

[1] Confirmed by Vladimirov & Danilina.

The conception of a late development of the glycogenic function of the liver is no new one. In 1858 Claude Bernard published his researches on mammalian embryos, in which he clearly showed that the glycogenic function later to be undertaken by the liver was, during the greater part of foetal life in mammals, carried out by the placenta. On p. 120 he wrote in a footnote, "Dans les oiseaux (poulet) j'ai constaté, avant le développement des cellules glycogènes du foie, l'existence de cellules glycogènes qui se développent dans les parois du sac vitellin; mais n'ayant pas pu suivre encore complètement leurs évolutions, je traiterai ce sujet dans une autre communication, me bornant aujourd'hui à parler des mammifères". This promised research was delayed for six years owing to Bernard's illness. On March 31, 1864, he deposited a "pli cacheté" at the Académie des Sciences, in which he stated that he had shown the presence of glycogen in the blastoderm of the chick, and regarded it as comparable, from this point of view, with the placenta of mammals. Moreover, he had isolated the glycogen from the blastoderm and identified it chemically, obtaining from it alcohol and carbon dioxide by appropriate fermentation. In 1872 Bernard read a full account of subsequent experiments before the Academy, and caused his sealed communication to be opened and read. His own words may be quoted, "4 Juin 1860. Sur un œuf de poule du deuxième au troisième jour d'incubation, j'ai détaché avec des ciseaux la membrane vitelline tout autour de l'area vasculosa; je l'ai enlevée avec des pinces de manière à appliquer sa face extérieure contre une lame de verre. En examinant ensuite sous le microscope cette preparation, j'ai vu très nettement des cellules glycogéniques et des granulations de glycogène qui prenaient une couleur rougeâtre par la teinture d'Iode acidulée avec l'acide acétique crystallisable". Bernard concluded that the blastoderm contained a notable store of glycogen for the needs of the embryo. He afterwards published a full account of his work in this field in his masterly *Leçons sur les phénomènes de la vie*.

It will be convenient here to discuss birds and mammals indiscriminately with reference to what Claude Bernard called the phenomenon of the "foie transitoire" or temporary liver. Bernard described in detail the histological appearances of the placenta when stained to show glycogen, but he did not rely solely on histochemical observations, for he mentioned the results of several chemical experiments which led to the same conclusion. "Il existe en effet," said Bernard,

"avant que le foie fœtal puisse exécuter ses fonctions, un véritable organe hépatique placentaire qui produit la matière glycogène." Bernard was for long puzzled by the fact that he could demonstrate this glycogenic function of the placenta with ease on guinea-pigs, rabbits, etc., but not on ruminants such as cows and sheep, but he eventually found that the reason for this was purely anatomical. In the case of the ruminants, the vascular and glandular components are quite separated, and, while the former continue to grow till term, the latter disappear by an atrophic degeneration. At birth, then, there may be very little left of the hepatically functioning part of the placenta. "One must add", said Bernard, "that all the time the amniotic placenta is increasing in size, the foetal liver possesses neither its adult functions nor its adult structure, while precisely from the moment that the foetal liver has attained an adult character and that its cells having acquired their definitive form, begin to secrete and store glycogen, the hepatic organ of the amnios begins to disappear." Bernard reported also that the cells of the skin, the intestinal mucosa, the mucous membranes of the respiratory and genito-urinary passages, the muscle-cells, etc., could be shown to contain supplies of glycogen in foetal life, and at a time when the liver was completely devoid of it. Glands and bony or nervous structures, however, were always free from it. This led to a series of researches on the glycogen content of tissues, mostly histochemical, which will be referred to later.

Immediately following Bernard's paper in the *Annales des Sciences Naturelles*, there is to be found a note by Serres entitled "Des corps glycogéniques dans la membrane ombilicale des oiseaux". Bernard's communication, he said, revealed to him the nature of those little glandular bodies which appear on the surface of the chick's blastoderm during incubation, and which he had figured previously without knowing what they were. "We see these little objects", he said, "from the twenty-fifth or thirtieth hour of incubation onwards. Their whitish colour suffices to distinguish them from the blood islands which have a reddish tint. At the thirty-fifth hour they become of a clear yellow colour and their increased size allows them to be more easily made out. . . . From the third to the sixth day their volume continues to increase but the proliferating arteries and veins partially hide them." About the 12th day they begin to disappear, and from histological considerations this time corresponds

with the arrival of the foetal liver cells at a stage suitable for the storage of glycogen. "May we not say", continued Serres, "that there exists in the case of the chick a diffused hepatic organ, or a transitory liver, analogous to that which M. Claude Bernard has just demonstrated in the placenta of ruminants?"

The work of Claude Bernard was for many years afterwards repeated and confirmed in a fragmentary manner. For the most part the work was histochemical, depending entirely on the iodine method. Thus Godet in 1877 described a "glycogen layer" in the rabbit's placenta. Langhans found none in the fully developed human placenta, though it was plentiful at earlier stages, as Merttens showed. Maximov, who made a very detailed study of the rabbit placenta, described the glycogen as increasing in amount up to a certain point, and then dying away. This was also observed by Ulesko-Stroganova and by Chipman, who, however, differed greatly between themselves as to the actual situation of the glycogen. Chipman never found glycogen in the foetal part of the rabbit placenta. It appeared in the maternal part first on the 8th day, and increased rapidly in amount, reaching a maximum between the 12th and 16th days. After that time it diminished steadily, and after the 22nd day there were only found small glycogen granules scattered in the midst of conglomerate masses of uni- and multinuclear cells, save in the zone of separation, where some cells remained distinct and retained their granules of glycogen. Glycogen was not found by Chipman in the foetal liver of the rabbit before the 22nd day, after which it increased rapidly and steadily till birth. He did not observe any diminution after birth. Other histochemical workers were Rouget; Marchand; Brindeau; and Schonfeld, who all reported first an increase and then a decrease in the glycogen of the rabbit placenta. Jenkinson; Gierke; and Saake worked on the mouse placenta, and published similar results. Driessen and Barfurth investigated that of the guinea-pig, Kajimura that of the bat, and Happe; Plesch; and Todyo that of man, from this point of view. A particularly interesting result was that of Külz, who confirmed Bernard's finding that glycogen was present in the 2-day-old chick embryo, but could not confirm the presence of glycogen in the cicatricula. The histochemical workers did not confine their attention to the placenta, and several of them, especially Barfurth, stated that in the early stages of development little or no glycogen was to be found in the foetal liver.

These conclusions were criticised by Pflüger in 1903 with all his usual vigour. He maintained that the histochemical reaction was liable to be misleading, as in instances where it had been negative for early embryos he had succeeded in isolating glycogen from them, and even estimating it quantitatively. Pflüger did a good service in drawing attention to the inadequacy of histochemical methods, and soon a number of investigations appeared in which chemical analyses were made.

Barfurth himself, for instance, published figures for a rabbit embryo liver and placenta, which are included in Fig. 278. Butte reported a value of 8·7 gm. per cent. glycogen for the embryonic liver of the dog at term and of 0·42 for the corresponding maternal liver. Paschutin found no glycogen in the livers of cow embryos 10, 14 and 21 cm. long, but isolated a certain amount from the liver of an embryo 40 cm. long. At birth McDonnell found 2 per cent. Demant found as much as 11·4 per cent. in the liver of a dog at birth, and noted that the quantity steadily decreased for several days afterwards (see Fig. 277). The cat embryo at term, however, has not such large amounts of glycogen in its liver, according to von Wittich, who only found 0·23 per cent. It is difficult to assess these early papers.

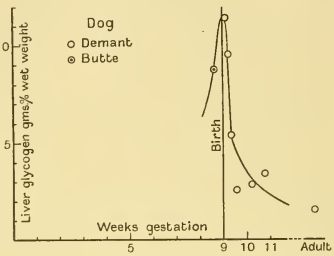


Fig. 277.

For the human embryo, von Wittich found 0·24 per cent. glycogen at 5·5 months development. A. Cramer found an average of 1·45 per cent. at term for liver glycogen, and 0·08 per cent. for placenta glycogen. The latter figure was fixed at 0·52 by Moscati, who found more in a placenta of the 7th month, and more recently at 1·06 per cent. by Clogne, Welti & Pichon, though Bottazzi could hardly find enough to estimate. Perhaps his placentas were not quite fresh, for Moscati showed that on standing at room temperature their glycogen content diminishes, and reaches nil 24 hours after the cutting of the umbilical cord. Adamov found 1 per cent. in human foetal livers.

In 1907 Mendel & Leavenworth studied the occurrence of glycogen in the embryo pig. Their results will be referred to again; here the important point to note is that they could never find any in the

liver before the stage of 23 cm. length, i.e. until the 110th day of development. Exactly analogous results were obtained by Zaretzki on the guinea-pig, from the liver of which no glycogen could be isolated till late in development.

Not until 1908, however, was the assumption of the glycogenic function by the liver put on a firm chemical basis. In that year Lochhead & Cramer made a very complete study of the movements of glycogen in the placenta and foetal liver of the rabbit.

It may be said at once that it led to a remarkable vindication of the views of Claude Bernard. Lochhead & Cramer applied the Pflüger method for glycogen to the embryos and placentas of rabbits. The maternal placenta of the rabbit can be divided into the following three parts: (*a*) that next the uterine muscle, which represents the plane of separation at the end of gestation, (*b*) an intermediate part, the region of the uterine sinuses, and (*c*) a part which extends up as a series of peninsulae between the foetal columns, which are analogous to the villi of the human placenta and were described by Chipman as ectodermic tubules with a plasmodial covering. Chipman found histochemically that there was a good deal of glycogen still left in (*a*) at the time of birth, but none in the zone of uterine sinuses and none in the region of peninsular projections, although both these were full of glycogen earlier. When the two parts of the placenta are pulled apart, the delicate peninsulae are left attached to the foetal placenta, so that the glycogen in the foetal part is really not foetal, but maternal. Thus by analysing the two parts of the placenta obtained by mechanical separation information was gained on the changes in glycogen of the two different parts of the maternal placenta. Lochhead & Cramer called the glycogen in the part of the placenta nearest the uterine wall the "distal glycogen", and that in the peninsulae and foetal placenta the "proximal glycogen". This latter portion would come principally from maternal tissue, though a small quota might be supplied from the true foetal part of the placenta. The percentage of distal glycogen they found to be quite comparable to that in the healthy adult liver, rising from the 14th day of development to the 18th, remaining constant from the 18th day to the 22nd, and then rapidly falling till birth. This is shown in Fig. 278. The proximal glycogen was very small in amount, reaching at its maximum only 11 mgm. per placenta as opposed to the 93 mgm. of the distal glycogen, and did not affect the curve of

total glycogen per cent. of the placental weight, except at the time (between the 18th and 22nd days) when the maximal placental amounts are present. These facts are in exact agreement with the work of Chipman.

Fig. 278 should be compared with Fig. 270. It can be seen that they are fundamentally alike—in the case of the chick the rising embryo curve and the falling non-embryo curve cross at the 17th day of development; in the case of the rabbit the rising embryo curve and the falling placenta curve cross at the 27th day of development. It is interesting to enquire whether these cross-over points occur at equal percentages of the whole developmental time, and it is easy to calculate what percentage of the total amount of glycogen in the system is at any given moment in the embryo and what percentage is in the adnexa. If this is done a graph is obtained like that in Fig. 279, from which it may be deduced that the mid-point in the assumption of the glycogenic function (the "cross-over point") by the embryonic liver occurs when 82 per cent. of the total development is achieved in the case of the chick and 91 per cent. in the case of the rabbit. Another interesting point which emerges from Fig. 278 is that the amounts of glycogen in the embryonic liver and the amounts in the placenta would not fall on a horizontal line if added together. This must mean either that some of the placental glycogen is destined for other organs of the embryo than the liver, or that a catabolism of carbohydrate as energy source is going on. The latter possibility fits in, of course, with what has already been said about the energy sources of mammalian embryos (see pp. 729 and 993).

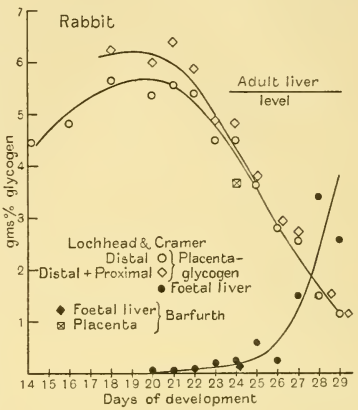



Fig. 278.

"The most obvious phenomenon in the decidual cells of the rabbit is the presence of glycogen at a time when the foetal liver cells store

only the merest traces", said Lochhead & Cramer. They found that glycogen could not be detected histochemically in the foetal liver before the 22nd day. A variety of diets had no effect on the amount of glycogen in the placenta and embryo, a finding quite in harmony with other work, which demonstrates the remarkable independence of the reproductive system against external influences. "The constancy of the amount of glycogen deposited in the placenta and in the foetal tissues generally", said Lochhead & Cramer, "contrasts markedly with the fluctuations in the glycogen store of the adult liver, both in the normal and in the pregnant animal, and shows up again the autonomy of the glycogen metabolism of the foetus." Some interesting experiments on the effect of phloridzin were also made by these workers. 0·6 gm. of phloridzin was injected daily into the mother animals from the 8th day of gestation onwards, the time when, according to Chipman, glycogen first appears in the maternal placenta. The result showed clearly that the placenta does not give up its glycogen readily to the maternal organism; thus on the 23rd day of gestation, a normal animal would have 4·5 per cent. of glycogen in its placenta and a phloridzinised one 4·24 per cent., or again 2·56 and 2·68 per cent. respectively. In some cases, however, there was an interference with growth, and the embryos were stunted; when this was so, the glycogen percentages were less than normal. As for the foetal liver after treatment with phloridzin, it had a lower glycogen content than normal, but yet much higher than the corresponding maternal liver.

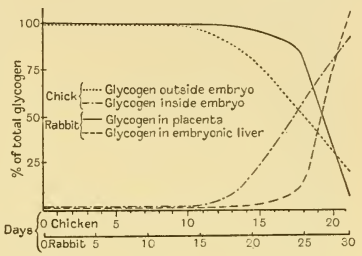



Fig. 279.

Since Lochhead & Cramer's classical investigations, the general inter-relations between the liver and the placenta laid down by them have been confirmed by a number of workers. Thus Clogne, Welti & Pichon found in human embryos that the liver glycogen increased from 1·8 gm. per cent. dry weight at the 3rd month to 29·5 gm. per cent. at the 9th month, while the placenta glycogen correspondingly decreased from 2·75 at the 3rd to 1·05 at the 9th

month. For this last value, Higuchi got a lower figure—0·032. Again, Loveland & Maurer found 100 mgm. of glycogen in rabbit placentas of 22 days' gestation and less than 25 mgm. at 32 days, and their histological checking fits in down to the last detail with what has been said above. Correspondingly Snyder & Hoskins reported that the glycogen of the rabbit foetal liver rose from a trace to 40 mgm. per gram—as much as the adult possesses—while the glycogen of the foetal body increased fivefold.

Interesting experiments have also been made by Huggett who has given attention to the factors which modify the amount of placental glycogen. These turned out to be few, for the percentage of glycogen in the placenta of the rabbit was unchanged by starvation, by carbohydrate feeding, by injections of carbohydrates, or by injections of hormones (adrenalin, thyroxin). Repeated injections of large doses of insulin did succeed in slightly lowering the percentage, and as phloridzin had already been shown by Lochhead & Cramer to have a similar effect, Huggett concluded that the only influences having any marked action on the glycogen of the maternal placenta were the profound disturbances of metabolism induced by massive insulin doses, phloridzin, and semi-pathological changes such as those induced by ether, amytal, and tetrahydro-$\beta$-naphthylamine[1]. Wertheimer has also made similar observations. All the glycogen of the maternal organism can be mobilised in the rat or guinea-pig by the action of cold and adrenalin, but the glycogen in the foetus remains untouched. This immunity persists for some time after birth, newborn animals requiring huge doses of adrenalin to shift any glycogen. It was significant that neoplasms also remained uninfluenced by treatment affecting the carbohydrate stores of the rest of the animal, and Wertheimer in other experiments (see Appendix II) found that amphibian ovarial eggs possessed a like independence.

We may now return to the development of the chick embryo, and unravel the mechanism of its carbohydrate metabolism further. Fig. 270 shows that the 11th day of incubation may be fixed on as being the point in ontogeny at which the glycogen storage in the embryonic body begins to become notable, when considered in absolute terms, or at which it is increasing more rapidly, if considered in terms of per cent. dry weight (see Fig. 280 taken from

[1] Conversely, placental glycogen cannot be increased by alimentary hyperglycaemia (Runge & Hartmann; Kessler).

Murray[1]). Both Mellanby and Schmalhausen have weighed the liver at different ages in the embryonic chick, but the 11th day is not associated with any specially marked change in weight. Nevertheless, that it is the embryonic liver which accounts mainly for the results obtained on the entire embryo appears strikingly from the careful

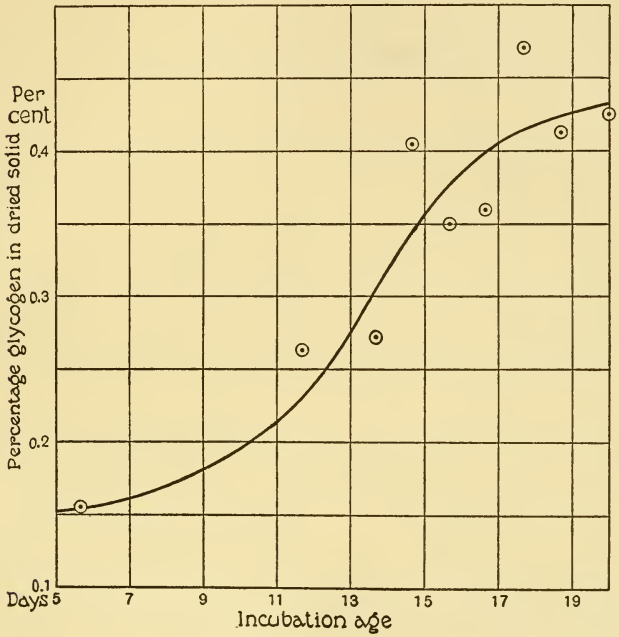



Fig. 280.

histochemical work of Potvin & Aron, who in 1927 examined the liver of the chick during each day of development, and could find no traces of glycogen in it until the 11th or 12th day. After the 14th day they reported that its increase was very rapid, a finding in exact agreement with the chemical results of Murray and Sakuragi[2],

[1] Vladimirov & Danilina's curve rises similarly but somewhat higher throughout.

[2] And of Vladimirov, who finds that between the 14th and 20th day of development the liver glycogen accounts for between 35 and 40 per cent. of the whole.

and a confirmation of some forgotten observations of Claude Bernard. Another rather abrupt change in the liver of the embryo chick was revealed by the investigations of Heaton, who observed a change in the biological properties of the liver cells on the 11th day. Before that time, its cells in tissue culture grow like epithelial cells (having arisen, of course, as a diverticulum from the gut), but after that time they grow like fibroblasts. Nor is this merely a morphological difference, for after the 11th day their growth is inhibited by yeast extract, just as that of fibroblasts always is, but this is not the case earlier. The change takes place regularly between the 11th and 12th days, i.e. just about the time when the curve for glycogen in the embryo shown in Fig. 280 inflects and rises sharply. In this connection it is significant that Holton found that the liver will not grow in chorio-allantoic grafts after the 11th day. Nordmann has studied the metabolic behaviour of explanted liver-cells. He states that glycogen (observed histochemically) is synthesised by all stages from the 9th day onwards. The earliest ones (9th day) showed the presence of glycogen very soon after explantation and retained it for nearly a fortnight in culture, the later ones (11th or 12th day) retained it only for about 5 days. This synthesis of glycogen seemed to be independent of the constitution of the medium.

Serres' histological observations, illustrated in the *Archives du Muséum d'Histoire Naturelle*, have since been confirmed by J. T. Wilson and by H. J. Allen. Allen, who seems to have been ignorant of the pioneer work of Serres, made a histochemical study of the glycogen in the yolk-sac of the chick at various stages. She found it to be present from the earliest time onwards, distributed all over the vascular area in the form of mahogany-coloured masses scattered among the cells. The head ectoderm, the heart, and the myotomes acquired glycogen very early also. "Apparently the yolk-sac", said Allen, "furnishes a way-station in which carbohydrates are stored as glycogen on their way from the yolk to the embryonic tissue."

Since the chick and the rabbit both exhibit the phenomenon of the "foie transitoire", the practice may be very general. It has been shown to take place in the ovo-viviparous selachian *Mustelus vulgaris* by Blanchard, who reported that the vitelline membrane contained abundant stores of glycogen. His methods were histochemical and the research was never published in detail.

The transitory liver may be regarded as an excellent instance of those special functions of embryonic life which will be discussed in the Epilegomena.

## 8·6. Free Glucose, Glycogen and Insulin in the Embryonic Body

It will be convenient now, before proceeding further, to ask what happens to the free carbohydrate of the embryonic body, for so far this important fraction has not been discussed at all. Only one set of measurements exists, those of Needham, but these were done on a very large number of embryos with the Hagedorn-Jensen method, and some reliance may be placed on them. The curve for absolute milligrams of free glucose per embryo, after having been suitably corrected for the presence of creatinine on the basis of the factor introduced by Holmes & Holmes (that 8 mgm. of creatinine affect the Hagedorn-Jensen reagents to the same extent as 1 mgm. of glucose), naturally rose, keeping pace with the growth in size of the embryo. It is shown in Fig. 281. It will be remembered that the total glucose in the embryo also rose steadily with the increasing size, as is shown by Fig. 264, but, if the two are compared, it will be seen that the shape of the curve is not exactly the same in the two cases, so that, when the two are related by expressing the free glucose in percentage of the total glucose, a peaked curve emerges (see Fig. 284).

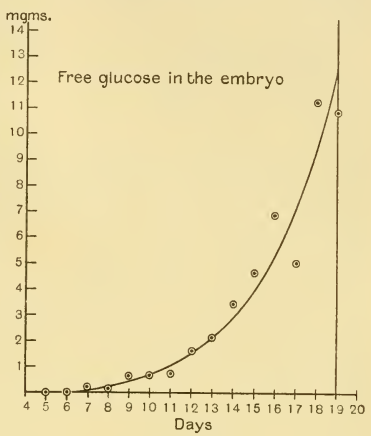


Fig. 281.

For the moment, however, attention may be directed to the relation between free and total glucose and wet and dry weight, as shown in Fig. 282 and Fig. 283.

We see that 100 gm. of embryo (wet weight) contain on the 5th day of development 160 mgm. of total carbohydrate and 8 of free

carbohydrate. The former falls to a level of about 100 and subsequently rises to over 300, the latter rises all the time in an S-shaped curve to about 48 mgm. This would seem to indicate that the carbohydrate present in the embryo on the 4th and 5th days, at which time there is more in proportion than at any subsequent period, is not in the form of free glucose, and as it cannot be glycogen it must be sugar in some unidentified combined form.

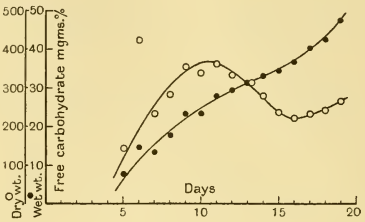



Fig. 282.

The dry weight data bear out this view even better. On the 5th day 100 gm. of dry embryo contain 3000 mgm. of total carbohydrate, about 100 mgm. of free carbohydrate, and about the same amount of glycogen. After that point the total carbohydrate continuously falls, reaching a value of 1750 on the 16th day, while the free carbohydrate continuously rises, its highest point being reached on the 11th day with a value of 360 mgm., after which it falls, but not below 220 mgm. The fact that the total glucose falls, while the free glucose rises, is of some interest. The presence of such a large proportion of glucose not free and not in the form of glycogen may be correlated with certain histological facts which have been known for some time, and which have already been referred to (see p. 566).

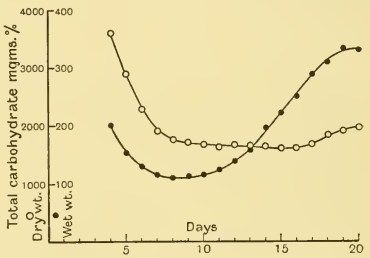



Fig. 283.

What happens to the concentration of the various carbohydrate fractions in the water of the embryonic body? It was pointed out above that when total protein, total carbohydrate and total fat were expressed as grams per 100 gm. of water in the embryonic body, the protein and the fat start at a very low level (owing to the great wetness of the earliest stages) and rise steadily, while the carbohydrate begins fairly high, falls to a minimum on the 8th day, and

thereafter rises with the others (see Fig. 221). This was interpreted as illustrating the importance of carbohydrate as an architectural material in the youngest stages, an importance which was, however, only transitory, and gave way before development was half complete to the predominance of protein and fat found in the adult. The data for the free glucose made it possible to determine to which of the carbohydrate fractions the total carbohydrate concentration curve owed its preliminary peak. Without doubt it is the great amount of mucoprotein glucose present in the embryo in the initial stages which causes this effect.

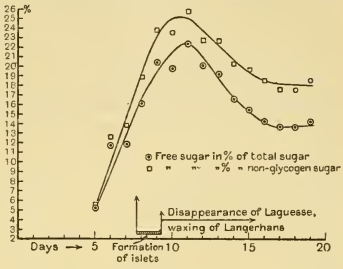



Fig. 284.

Since we know the glycogen present in the embryo each day, a glycogen/glucose ratio can be constructed. As Fig. 285 shows, it remains steady till the middle of incubation, after which it rather suddenly rises, and acquires a new steady value. The point which is important here is the peak in the curve representing the free glucose in percentage of the total glucose, and, at exactly the same time, the rapid change in the glycogen/glucose ratio. Why should there be a higher proportion of free glucose in the embryo then than at any other time? The answer must surely be that the free glucose goes on increasing until a point is reached at which the production of insulin overhauls it and a control of the proportion of free and combined sugar can be attained. Fig. 285 shows diagrammatically the part played by the pancreas in this mechanism. In early embryonic life there are one and a half times as much glucose as glycogen, but after the critical 11th day there are one and a half times as much glycogen as glucose. In this way the embryo rather quickly approximates to the adult condition, once the time has come

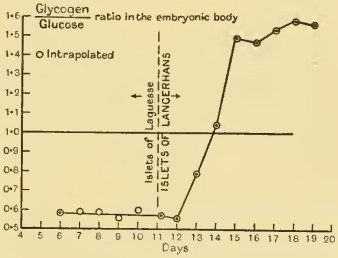



Fig. 285.

for it to do so, for a preponderance of glycogen over free glucose may roughly be taken as characteristic of the mature animal. Another instance of such a comparatively rapid passage from the embryonic to the adult condition will be seen in the nitrogen partition in the urine of the chick (Section 9·5).

It is interesting to note that the effect of insulin on the free glucose curve seen in Fig. 284 is a phenomenon revealed in the intact organism following its usual courses and not subject to the abnormalities necessarily consequent upon depancreatisation or other experimental treatment. Another point is that the appearance of insulin occurs in that portion of embryonic life in which development is irrevocably determined and self-differentiation is going on. During the early stages, when a great degree of regulation is possible, the embryo has no insulin, and the hormone only arises after chemodifferentiation has taken place and the fate of every cell is finally determined. As will appear in Section 15, good evidence also exists that this applies to other hormones besides insulin, and probably to all of them.

Hanan demonstrated that insulin hypoglycaemia can be produced during the last week of development. Taking 14–16-day White Leghorn embryos, he injected insulin into the air-sac, and then, withdrawing blood from the allantoic vein at its bifurcation just below the air-sac, estimated the blood sugar by the Hagedorn-Jensen method. From the normal value of 209–296 mgm. per cent. a marked lowering could be observed. If glucose was injected into the air-sac instead of insulin, the blood sugar rose, and it could also be made to rise by bleeding the egg. Riddle's finding that adult birds will survive a dose of insulin thirty times as strong as that which would kill a rabbit was extended by Hanan to this later period of incubation.

In 1922 Aron, as the result of comparative studies on the embryos of the sheep, guinea-pig, pig, and man, made the generalisation that the glycogenic function of the liver (as judged by the accumulation of glycogen within it) always occurred at the moment when the interstitial portion of the pancreas was taking on its adult histological appearance. His experiments did not include the determination of the glycogen content of the placenta, but he analysed the foetal liver at different stages of development, and obtained the curves shown in Fig. 286. "The glycogenic function of the liver", said Aron, "manifests itself at a fixed time in ontogeny. Its installation varies in different

species, for with the sheep it appears early, at the end of the second month of gestation, but does not rise much till the end of the fourth month. With the pig it appears suddenly at the beginning of the last fortnight of gestation. With the guinea-pig it appears at the 40th day of gestation but does not rise very fast." Aron was not inclined to accept the simple hypothesis of Claude Bernard that the glycogenic function is taken on by the liver as soon as the cells are morphologically ready to receive it. He regarded it as much more likely that the passage of this function from placenta to liver was under the control of an endocrine agency, and was thus led to examine the behaviour of the pancreas. Laguesse had already pointed out that the appearance of the pancreatic islets in early embryonic life was quite different from that which they presented later, and Aron extended his work by showing histologically that the second generation of islets, i.e. the islets of Langerhans, appeared rather rapidly out of the islets of Laguesse just about the time when the glycogenic function was installing itself in the liver. This relationship held quite rigorously as between the different animals; thus in the sheep the islets of Langerhans appeared first about the 50th day, while in the pig they did not appear till the 100th day. The significance of these findings can be seen from Fig. 286. Aron also brought various lines of evidence together to show that the Laguesse islets produce no insulin, as those of Langerhans do. The transformation of the former into the latter may be very sudden. "Cette véritable explosion endocrinienne", said Aron, "peut se déclancher un peu plus tôt ou un peu plus tard selon les cas. Quoi qu'il en soit, on observe constamment une coïncidence frappante entre la présence de nombreux îlots de Langerhans dans le pancréas, et le dépôt dans la foie d'une notable quantité de glycogène." Even in the case of man, where Aron only worked out the appearance of the

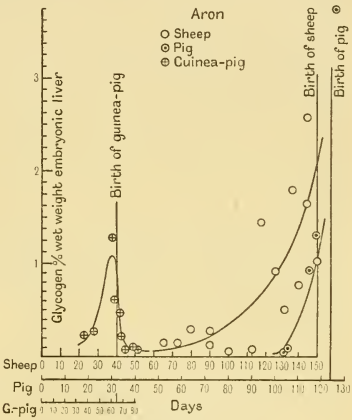



Fig. 286.

Langerhans islets, the relation holds, for the change occurred at the beginning of the 4th month of gestation, and from the work of Livini we know that no appreciable quantity of glycogen can be shown to be present in the liver before that time. Gierke and Lubarsch both made similar statements about the liver in early pig embryos. Thus the biliary always appears in ontogeny before the glycogenic function of the liver.

In 1928 Aron extended these conceptions to the amphibian embryo and larva. Glycogen was determined by histochemical methods in the livers of *Rana temporaria*, *Rana esculenta* and *Bufo vulgaris*. Aron found, just as Claude Bernard had found long before, that none is present before the appearance of the hind limb buds, i.e. before the disappearance of the yolk. Complete ablation of the pancreas in early stages altogether prevented the appearance of the glycogenic function, so Aron naturally concluded that the mechanism of its control in amphibia was similar to that in mammals. This would mean that the chemical regulation of the embryonic and yolk-sac period in the frog is carried on without the aid of insulin. Aron has suggested various further mechanisms of control involving the action of the thyroid, but these are not at present very certain.

Judging from the work of Goldfederova, the increase in liver glycogen, when it does come, must be very sudden, for she obtained the following figures:

| Stage | Glycogen % wet weight: Liver | Muscles |
|---|---|---|
| Hind limb buds visible ... ... | 11·9 | 0·05 |
| Mobile hind limbs and tails ... | 9·7 | 0·16 |
| Front limb buds visible ... ... | 9·6 | 0·20 |
| Resorption of tail ... ... ... | 10·7 | 0·35 |
| Completely metamorphosed ... | 8·6 | — |

Claude Bernard himself went further afield than to the amphibia, for in studying the embryos of molluscs, especially the common oyster, he observed that the cushion or disc which carries their cilia and makes them mobile was very rich in glycogen (histochemically). As the disc subsequently falls off when the embryos become sessile, Bernard felt justified in seeing in it an analogy with the placenta of mammals as regards glycogen storage.

In other researches Aron, Stülz & Simon carried out the experiment of Carlson & Drennan at various stages of gestation in the dog, i.e. they depancreatised the mother, and observed whether there was any evidence of protection from hyperglycaemia due to

insulin from the foetal pancreas. Such experiments are open to various criticisms on grounds of technique, and in Section 21·8 we shall examine them in more detail. They found that up to the 7th week there was no protection but that it was demonstrable afterwards. This agreed chronologically with the time at which the transformation of Laguesse islets into Langerhans islets was going on, and supplied more evidence that the former were incapable of secreting insulin. Then Potvin & Aron investigated the islet tissue in the pancreas of the chick. They found that the first islets appear on the 8th day of development, and for a day or two those of Laguesse predominate, but these soon give place to Langerhans islets, so that by the 15th day there are none of the former left. It is impossible not to be struck with the correlation between these histological and physiological data, and the biochemical evidence of Figs. 284 and 285.

## 8·7. General Scheme of Carbohydrate Metabolism in the Avian Egg

A general scheme of carbohydrate metabolism in the developing chick may therefore be provisionally summarised as follows:

(*a*) *Zero hour of development till the 7th day.* The carbohydrate in the embryo at the earliest stages, i.e. about the time when the yolk is first completely enclosed by the blastoderm, is to a very large extent composed of glucose in combination with protein. Glycogen increases outside the embryo, being stored in the yolk-sac and parts of the blastoderm. Free sugar increases steadily within the embryo both absolutely and relatively, being uncontrolled by the pancreatic hormone. The liver and pancreas arise as epithelial buds from the wall of the duodenum on the 3rd day. Glucose combustion predominates.

(*b*) *Eighth to 11th day.* The importance of the mucoprotein fraction of the glucose in the embryo diminishes, but the free glucose continues to rise proportionately, as does the glycogen in the yolk-sac and blastoderm. At this time 90 per cent. of the egg's glycogen is outside the embryo. The liver and pancreas change their growth, the former increasing in bulk and the latter developing the islets of Laguesse. By the 10th day the islets of Langerhans are in evidence, and insulin is being secreted in increasing amounts. At this time also a certain extra quantity of fat enters the embryo from the yolk, and is there transformed into carbohydrate.

(*c*) *Eleventh day.* The insulin about this time attains such concentration or activity that it is able to stop the relative increase of free glucose, which now passes through its peak. Perhaps the appearance of insulin is sudden, for Pucher & Hanan could not find any in the embryo until the 11th day. The liver cells, changing the character of their growth, prepare to receive stores of glycogen, and from this point onward do so (Murray; Sakuragi; Potvin & Aron).

(*d*) *Eleventh day till the end of embryonic development.* The control of the free glucose by the insulin continues, so that as per cent. of the total glucose it declines. Synchronously with this process the glycogen of the embryonic liver increases steadily in amount, its origin being partly the glycogen of the transitory liver in the blastoderm and partly the diminishing free glucose. In other tissues also, e.g. the intestinal walls (Maruyama) glycogen steadily increases. The carbohydrate systems in the chick are now fully sensitive to the action of insulin.

The concomitant events outside the embryo may be traced by the curves given above. The most remarkable items seem to be that, when the free carbohydrate is at a maximum in the embryo, it is at a minimum outside, and that the periods of maximum importance of mucoprotein glucose inside the embryo coincide with the periods of maximum catabolism of ovomucoid outside.

## 8·8. Embryonic Tissue Glycogen

A good deal has been said already about the glycogen in the foetal liver at different stages, and the question now arises as to what happens to the glycogen in the rest of the body and its different parts. Fig. 270, embodying the data of Murray and Sakuragi, demonstrates that in the case of the chick the glycogen in the embryonic body as a whole rises in a regular curve both absolutely and relatively. In the last half of last century, much importance was for some reason attached to the embryonic glycogen, and it was thought that this substance was in some mysterious way connected with growth and differentiation. This belief died out when it came to be found that glycogen is not present in embryonic tissues to a greater extent than in adult ones. Fig. 287 shows the combined data of various investigators, the abscissa being in all cases conception age. Gage claimed to have seen glycogen histochemically in pig embryo

brains, but Mendel & Leavenworth could not find any there, thus confirming Bernard, although they readily obtained it from the

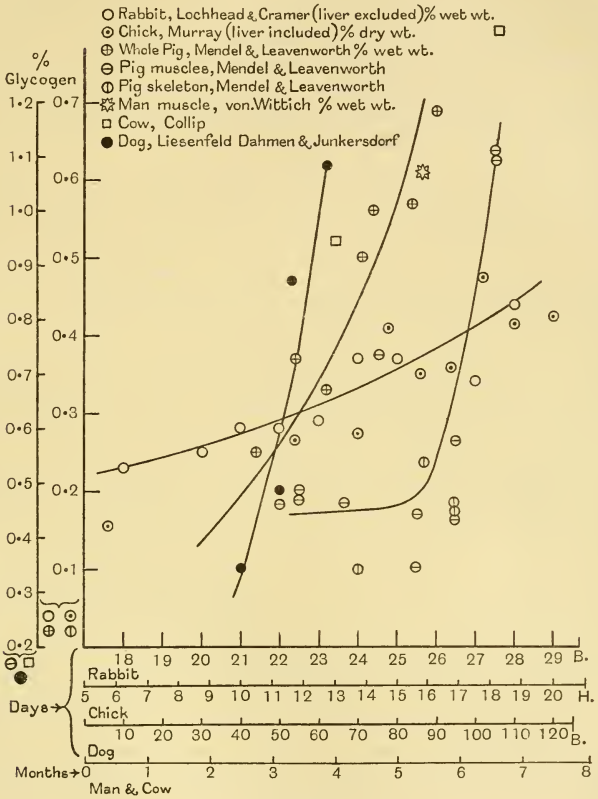



Fig. 287.

muscles and skeleton. It is evident from Fig. 287 that the embryonic tissues show no special richness in glycogen.

In spite of these chemical facts an enormous quantity of labour has been expended by investigators who have charted out histo-

chemically the various regions of the embryo according to the depth of colour obtained with iodine and other reagents. The two most outstanding surveys of this kind are those of Creighton and of Sundberg, in whose papers will be found lists of tissues and organs microscopically rich and poor in glycogen respectively. Creighton introduced the theory that glycogen "acts in the embryo as the precursor or deputy of haemoglobin until such time as the vascularity of the part is sufficiently advanced, and in other cases as the substitute of haemoglobin from first to last, i.e. in those tissues which are built up in whole or in part without the direct access of blood". Creighton also thought that "the cartilages which are destined to continue throughout life as cartilages have little or no glycogen in the foetal period, but those which later will ossify have plenty and it usually appears in the spots which afterwards become ossification centres".

Nobody now accepts Creighton's views and the attribution of any special embryological importance to glycogen is superfluous. While it may be useful to know the histological distribution of glycogen in the embryo, at present little physico-chemical meaning can be attached to most of this work. Investigators continue to labour along these lines, however, e.g. Ellis; Gragert; Glinka; Gierke; Gage; Lubarsch; Togari; Jordan. Livini has published a series of papers on the glycogen distribution in human embryos. None can be demonstrated in the liver till the end of the 2nd month. The muscles, lungs, skeleton and epithelial tissues begin then to acquire it; the pancreas, salivary glands, thyroid, parathyroid, thymus, suprarenal medulla, kidneys, smooth muscles, testes, ovaries, etc., have very little, and it appears irregularly, while the central nervous system, the suprarenal cortex and the retina never have any at all. Livini found that, as the liver glycogen rises towards the end of gestation, it falls in the organs of the second class, so that an additional source, other than the placenta, may be envisaged. All Livini's work is histochemical. It is clear that the glycogen of the body as a whole rises during embryonic life (Murray for the chick, Lochhead & Cramer for the rabbit, Mendel & Leavenworth for the pig, and Aron for the cow and the sheep). The only contradictory piece of evidence is contained in a dissertation by Kistiakovski, who stated that the quantity of glycogen in embryo cows and sheep diminished gradually until birth. This publication is not available in England, but it can probably be disregarded in view of the consensus of opinion.

Some other experiments on glycogen in embryonic life are worth mentioning. Driessen's histological work showed that the early ovum of the rabbit, the mouse, and man (from the 3rd to the 6th week in the latter case) is surrounded by a layer of cells very rich in glycogen. This is probably significant for the nourishment of the embryo, and brings to mind the observation of Zavattari that the test cells of ascidian eggs are full of glycogen granules also. M. R. Lewis studied the cells of early embryos of the chick and the minnow in tissue cultures. Glycogen was always present according to histochemical test in the latter case, but only for the first 48 hours of development in the former. This is in agreement with Külz's work already mentioned, where 5000 6-hour cicatriculae were taken and worked up for glycogen.

Another analogous line of investigation is that of Vastarini-Cresi, who has studied histochemically the appearance and distribution of glycogen in the chick embryo. The first traces appear, according to him, in the heart at the 2nd day of development, i.e. at the beginning of pulsation. He considers that the canalisation of vessels and spaces is specially associated with glycogen metabolism. He divides organogenesis into three phases: (1) Active cell multiplication in all directions to form a bud; here no glycogen can be found in them. (2) A period of glycogenic infiltration followed by one of canalisation or cellular dissolution. The inside cells become so full of glycogen that they cytolyse, and so form cavities. (3) Removal of the glycogen to other places.

## 8·9. Embryonic Blood sugar

The question of embryonic blood sugar and what happens to it must now be taken up. As long ago as 1875 Moriggia gave figures for foetal blood sugar in many animals.

Hanan's figure for the embryonic blood sugar of the chick at the 15th day is not in agreement with the later one of Vladimirov & Schmidtt, who also used the Hagedorn-Jensen method, but probably worked on chicks of a different breed. They found a constancy until just before hatching, and considered that from the 11th day onwards insulin was an effective agent in regulating the blood sugar level[1] (see

[1] Vladimirov has also studied the effect of adrenalin, asphyxia, etc. on the embryonic blood sugar of the chick. The results of Riddle & Honeywell agree with Vladimirov & Schmidtt rather than with Hanan, for they found the blood sugar of the embryo to be always less than that of the adult (3 species of pigeons).

Fig. 288). The blood sugar, however, is not a large enough factor to affect to any great extent the free glucose of the embryo as a whole, as a rough calculation demonstrates. Assuming 230 mgm. per cent. for the 20th day, the amount of blood in the embryo would be 1·54 gm. (from the formula of Dreyer & Ray, i.e. $B = \frac{W^{\frac{2}{3}}}{K}$, taking the mouse value (6·7) for $K$ as the mammal nearest in weight to the hatching chick). This would give 3·53 mgm. of blood sugar, which, although a high estimate, is only 15 to 20 per cent. of all the free sugar in the embryo.

The results which have been obtained on mammalian embryos are slightly more coherent. Aron investigated the blood sugar of embryos of the guinea-pig, rabbit, dog, cow and pig, using the Fontés-Thivolle method and accumulating the results shown graphically in Fig. 289. Although the points arrange themselves roughly along definite curves, Aron did not treat them as if this was so, but simply averaged out the data, and concluded that for each species of embryo there was a characteristic blood sugar level which did not change appreciably throughout intra-uterine life. The values thus calculated were as follows:

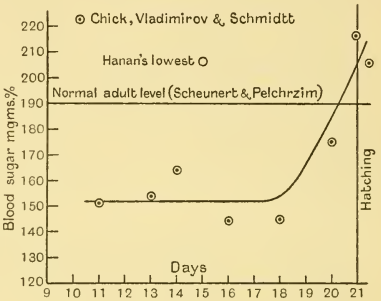



Fig. 288.

| Species | Blood sugar in mgm. % | |
|---|---|---|
| | Foetal | Maternal |
| Guinea-pig ... ... | 60 | 107 |
| Rabbit ... ... | 36 | 125 |
| Dog ... ... ... | 65 | 110 |
| Cow ... ... ... | 110 | 100 |
| Pig ... ... ... | 139 | 100 |

Thus in some cases the glucose concentration in the foetal blood was higher, in other cases it was lower, than in the maternal blood. For comparison, the following values of other observers may be advantageously placed here.

| Investigator | Species | Blood sugar in mgm. % | |
|---|---|---|---|
| | | Foetal | Maternal |
| Olow ... | Man | 80 | 120 |
| Rowley ... | Man | 90 | 110 |
| Hellmuth ... | Man | Foetal blood sugar always lower than maternal by 9–84 mgm. % | |
| Morriss ... | Man | 115 | 132 |
| Bergsma ... | Man | Foetal blood sugar the same as maternal at birth | |

These will be dealt with in detail in the Section on placental permeability. At present we must neglect the apparently regular changes in Aron's data and assume, with him, that the embryo is able to regulate its own blood sugar, for that was the point on which he laid special emphasis. "Le milieu intérieur du fœtus", he said, "reste, au point de vue de sa teneur en glucose, indépendant du milieu intérieur de la mère. Tout se passe donc comme si le fœtus réglait sa propre glycémie."

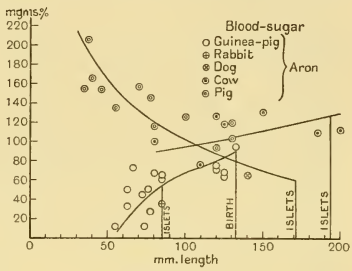


Fig. 289.

As we have already seen, Aron established the fact that the appearance of the islets of Langerhans in the foetal pancreas and the installation of the glycogenic function of the liver are synchronous events in the life of the embryo. Aron now found, working with dogs and cats, that, although the islets of Langerhans appear in the former case not till after the 7th week of gestation and in the latter case well before this time, their appearance was always marked by a greater independence of the foetal circulation. Before their appearance, the removal of the maternal pancreas led to an elevation of the maternal blood sugar which was reflected very closely by a rise in the foetal blood sugar, but afterwards, this correspondence was not nearly so marked. Thus at the 6th week in the dog, after removal of the maternal pancreas, the blood sugar was 360 mgm. per cent. in the mother and 340 mgm. per cent. in the foetus, but at the beginning of the 7th week, when a similar experiment was tried, the values were respectively 274 mgm. per cent. and only 175 mgm. per cent. Although the protection of the embryonic insulin was not complete, yet the embryo was clearly defending itself

against the diabetic state of the mother. Aron therefore divided the regulation of the foetal blood sugar into two periods, (*a*) an early one in which regulation depends solely on the function of the placenta, and (*b*) a later one in which it depends on the function of the insulin-producing islets of Langerhans as well. The problem of whether in the first of these two periods there is any control of the foetal blood sugar by the maternal insulin, Aron can hardly be said to have solved. Injections of insulin into the mother during the first period certainly lowered the foetal blood sugar, but not as much as the maternal, i.e. 20 to 25 per cent. of the normal instead of 50 per cent. or more of the normal. The conclusion seems justifiable that in period (*a*) the foetal blood sugar is kept by the placenta at a definite relation with the maternal blood sugar, and this may fall or rise as the latter falls or rises. Aron's conclusion was more complicated. He thought that the foetus, in normal conditions, received from the mother a utilisable form of glucose which it adapted to its own metabolism, and that, when the maternal pancreas was removed, this form of glucose was no longer produced, so that a foetal diabetic state would follow immediately upon a maternal diabetic state. It is difficult to see why this should have followed from his experiments. For further description of the work of Aron and his collaborators on the carbohydrate metabolism of the mammalian embryo, see Section 15·3.

Returning now to Fig. 289 it is obvious that the blood sugar falls in some cases and rises in others as development proceeds. As far as Aron's work went, it appeared to fall in the case of the pig, and to rise in those of the guinea-pig, rabbit, dog and cow. Further points were collected for the rabbit by Snyder & Hoskins but cannot be plotted, as the full data have not been published. These workers state that the foetal rabbit blood contains 33 mgm. per cent. at 22 days of gestation and 100 mgm. per cent. at 32 days—it thus rises and approaches the maternal level. Further work on foetal blood sugar levels is urgently required, especially in view of the interesting work of Scott, who in comparing the blood sugar of various mammals, found that it was highest in the smallest ones, i.e. those whose energy expenditure would be greatest (e.g. rabbit 107, guinea-pig 118, rat 138, mouse 245 mgm. per cent. among rodents).

The effect of glucose in the medium of embryonic cells in tissue culture was first studied by M. R. Lewis who found that its presence

was essential and that lack of it led to vacuolation and death in cultures of connective tissue cells from 8-day chicks. Willmer, using intestinal cells from 11-day chicks, confirmed this and found that the optimal concentration of glucose was rather lower than 1 per cent., though growth would sometimes proceed well in concentrations up to 2 per cent. When, however, Holmes & Watchorn came to make cultures of the embryonic rat kidney, they found that the optimal concentrations were much lower, varying around 0·2 per cent. or even a good deal less, and they suggested that the difference lay between avian and mammalian tissue. They recalled that the amount of glucose in the egg is at certain times quite large, and thought it likely that the chick's tissues might be exposed to greater concentrations of glucose in the egg than those of the rat in the uterus. As will be mentioned in the Section on protein metabolism, Holmes & Watchorn were able to show that glucose exerted a marked protein-sparing action on rat embryonic kidney tissue grown *in vitro*, as judged by the diminution in ammonia and urea-production.

Krontovski & Bronstein, who also worked with embryonic tissues from the rat, were able to demonstrate by microchemical methods a disappearance of glucose from the medium in which the cells were growing[1].

## 8·10. Carbohydrate Metabolism in Amphibian Development

We may now consider the movements of the carbohydrate fractions in eggs which have so far been left out of account. The reptiles have been very little investigated, but according to Tomita the 100 mgm. per cent. of free glucose present in the egg of the sea-turtle, *Thalassochelys corticata*, have disappeared entirely by the 30th day of development. Corresponding with this diminution there is a peak in lactic acid content (see p. 1053).

As regards amphibia, a histochemical study of the glycogen in the frog embryo and larva was made by Konopacki and by Konopacki & Konopacka. After fertilisation and the formation of the perivitelline space, the amount of glycogen thus estimated diminishes considerably, and apparently undergoes no further changes till the gastrula stage. At this time there is little to be seen; only a few cells of the

[1] This was confirmed by Holmes & Watchorn and by Friedheim & Roukhelman. Acidification of the medium may occur owing to the lactic acid produced (Magath).

blastula and gastrula have any in them. At the neurula stage, however, while the first organs are being formed, glycogen appears again, and rises in amount until it can be found in all the tissues all over the body. In later periods all organs do not behave in the same way, for in some of them the glycogen soon disappears again, while in others it steadily accumulates during larval life. Thus in the mesenchyme cells, the central nervous system and the eye-cups, its appearance at the neurula stage is only transient, and there is none there by the time that a length of 5 mm. is attained, but it persists much longer in all the epithelial cells, and definitely increases in amount in the skeletal and cardiac muscle. It will be observed that these findings are roughly in accord with those of Claude Bernard. Konopacki also studied the effect of the formation of the perivitelline space on the glycogen in the frog's egg, and found that it corresponded with a marked diminution of it—"le glycogène", he said, "disparaît presque entièrement", but the contents of the perivitelline space gave a very strong reaction for glycogen, so that it would not appear to be lost from the system as a whole.

Thus the glycogen in the frog's egg at fertilisation found by Kolb; Luchsinger; Athanasiu; Bleibtreu; and Kato (see Table 46 and Appendix II), is partly eliminated into the perivitelline space and almost wholly used up by the cells of the embryo before gastrulation. After that time more glycogen is formed and stored in the tissues, which after a preliminary general distribution, hand over most of it to the keeping of the liver and the muscles. The details were filled into this bare outline by the long memoir of Konopacki & Konopacka. Thus they showed that, after gastrulation, glycogen does not in general appear in endodermal cells, e.g. liver and pancreas, though the gill region is an exception. In the ectoderm it appears profusely, but does not persist there except in the epidermis, where it exists even after hatching. The organs of mesodermic origin take an intermediate position, for after gastrulation, glycogen is found to a considerable extent in them, but very soon disappears. The skeletal and cardiac muscles form an exception to this rule. No glycogen seems to be present at any time in the genital cells. During the hunger period after the yolk-sac has been all used up, if no food is provided for the larvae, glycogen disappears from all the places where it is stored. Conversely, if food is given, the glycogen stores increase throughout, and glycogen appears for the first time in the liver, the white matter

of the central nervous system, the cells of the choroid plexus and the retina.

Konopacki & Konopacka insisted on the necessity of histochemical work as adjuvant to purely chemical investigations, and, as it is true that chemical analyses at present cannot distinguish between regions such as the three germ layers, for instance, they were quite right. But what they did not emphasise was the fact that histochemical methods are much more uncertain than purely chemical ones.

Fauré-Fremiet & Dragoiu paid some attention to the glycogen in the developing frog's egg. Using the Bierry-Gruzevska method, they found 3·31 gm. per cent. (wet weight) glycogen in the unfertilised egg, or 7·81 per cent. dry weight, and in absolute figures 0·135 mgm. per egg. At hatching the glycogen had diminished to 1·75 per cent. wet weight, or 0·079 mgm. per egg, so that between fertilisation and hatching each egg had lost 0·056 mgm. of glycogen. It would thus appear that over the whole period there is a loss of glycogen, amounting to 41 per cent. of the amount originally there. Evidently the mechanisms at work in the frog's egg differ considerably as regards glycogen from those at work in the hen's. The fall in glycogen added to the fall in fat was found by Fauré-Fremiet & Dragoiu not to account for the fall in dry weight and calorific value, so they postulated a fall in protein as well. At the end of the larval yolk-sac period, these workers could find only traces of glycogen. Over the whole period a dry weight loss of 17·3 per cent. was observed, of which 3·2 per cent. was contributed by glycogen, for Fauré-Fremiet & Dragoiu did not envisage the possibility that the glycogen might have been transformed into some other substance.

This, however, was found to happen by Needham. Table 129 gives the results obtained, setting side by side with them the data of all the observers who have ascertained the loss in dry weight and the big gain in wet weight which the frog embryo has before hatching. It can thus be seen that, although Fauré-Fremiet & Dragoiu found that the egg has only 58·5 per cent. of its glycogen left at the end of development, it has 92·8 per cent. of its total carbohydrate, and the conclusion must be that the glycogen has been transformed into some other kind of carbohydrate, not, as Fauré-Fremiet & Dragoiu thought, that it has been combusted to provide energy for the embryo.

The balance sheet of the frog's development given in Table 129 shows that the average frog embryo gains about 21 per cent. in wet weight, and loses about 32 per cent. of its dry weight, while 41 per cent. of its glycogen disappears, but only 7 per cent. of its total carbohydrate.

Table 129. *Balance sheet of the developing frog embryo* (Rana temporaria).

| Wet weight (mgm.) | | | Dry weight (mgm.) | | | Glycogen (mgm.) | | | Total carbohydrate (mgm.) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 day | 8 days | Diff. | 0 day | 8 days | Diff. | 0 day | 8 days | Diff. | 0 day | 8 days | Diff. | Investigator | Method used |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 4·16 | 5·22 | + 1·06 | 1·9 | 1·65 | −0·35 | 0·135 | 0·079 | −0·056 | — | — | — | Fauré-Fremiet & Dragoiu (1923) | Bierry-Gruzevska |
| 3·99 | 4·42 | + 0·43 | 1·63 | 1·34 | −0·29 | — | — | — | — | — | — | Fauré-Fremiet & Vivier du Streel (1921) | — |
| 5·9 | 21·1 | +15·2 | 1·3 | 1·2 | −0·10 | — | — | — | — | — | — | Bialascewicz (1908) | — |
| — | — | — | 1·38 | 1·30 | −0·08 | — | — | — | — | — | — | Bialascewicz & Mincóvna (1921) | — |
| — | — | — | 2·33 | 1·15 | −1·18 | — | — | — | — | — | — | Bonnet & Barthélemy (1926) | — |
| 3·3 | 5·4 | + 2·1 | 1·36 | 1·05 | −0·31 | — | — | — | — | — | — | Williams (1900) | — |
| — | — | — | — | — | — | 0·050 | — | — | — | — | — | Haensel (1908) | Pflüger |
| 6·68 | 8·09 | + 1·41 | 2·13 | 1·51 | −0·62 | — | — | — | 0·0402 | 0·0373 | −0·029 | Needham (1927) | Hagedorn-Jensen |
| Average Result | | +4·62 (20·8 %) | — | — | −0·65 (32·4 %) | — | — | −0·056 (41·5 %) | — | — | −0·029 (7·2 %) | | |

The amount of total carbohydrate found, using the Hagedorn-Jensen method, is rather less than the amount of glycogen found by the Bierry-Gruzevska method, though very little less than the amount of glycogen found by the Pflüger method. This contradiction does not invalidate the arguments given above, for the values in each case are probably relatively exact. It is also likely that the total carbohydrate estimations are nearer to the absolute values than the glycogen ones, for the estimation methods for glycogen have never been very good, and are still under discussion. Thus Asher & Takahashi have criticised Pflüger's method severely, and the newer method of Rona & van Eweyk, which unfortunately nobody has used on the frog embryo, gives much lower results than the older ones.

On the whole, therefore, the results of the chemical investigators agree very well with those of the histochemical ones. But it is wrong to conclude, when glycogen is seen to be disappearing histochemically, that it must be destined for combustion purposes. It may only be

going to other forms of carbohydrate. This is one of the ways in which hurried conclusions from merely histochemical evidence may lead to confusion. It also shows again that glycogen cannot be considered as representative of the carbohydrate group.

One point which has resulted from these investigations of the carbohydrate metabolism of the frog embryo is that there is no glycogen in the liver until a very late date, so that Aron's views on the assumption of its function are strongly supported. Another point of interest is the probability that the hatched tadpoles derive nourishment from the jellies around their eggs. We have already seen that there is some evidence that they do (p. 909). If this is the case, the stores of carbohydrate in the form of mucoprotein there may be an important source for the sugar of their bodies, especially as they can absorb substances through their skins. It is clear that the question of amphibian carbohydrate metabolism has so far only been touched on, and that there is much room for an extended investigation of it, including the determination of glycogen and free glucose by improved methods on each day before and after hatching as well as on parts of the animal.

## 8·11. Carbohydrate Metabolism of Invertebrate Eggs

A certain amount of work has been done on the carbohydrate metabolism of the eggs of the nematode worm *Ascaris*. Brammertz and Marcus, using purely histochemical methods, observed a diminution of the glycogen in the eggs following fertilisation. Then Fauré-Fremiet estimated the glycogen before and after segmentation by the Pflüger method, obtaining 1·75 per cent. dry weight before and 1·05 per cent. dry weight afterwards, i.e. a loss of 0·7 per cent. over the whole period. On the other hand, he obtained a figure of no less than 21 per cent. dry weight for the ripe ovary, most of which must have been in the eggs. Von Kemnitz reported histochemical observations which showed that, in the ovaries before the eggs were laid, they possessed large stores of glycogen. Immediately after fertilisation, however, Fauré-Fremiet found only 4·67 per cent. of glycogen, though the chitin of the newly formed membranes accounted for an additional 9·23 per cent. The two added together did not equal the glycogen-content of the ripe ovarial eggs, from 7 to 9 per cent. being missing. "This quantity", said Fauré-Fremiet, "has disappeared without leaving any traces, and as we know that

the life of *Ascaris* is essentially anaerobic, the lost glycogen not transformed into chitin cannot have been burnt. Weinland showed that in *Ascaris* glycogen can be transformed into lower fatty acids such as butyric and valerianic." Unfortunately, this is now quite discredited (Slater). After fertilisation, the disappearance of glycogen continues slowly, the percentage dropping from between 4 and 5 to rather less than 2, and during segmentation this again falls to about 1. It will be remembered that in Section 1·12 (p. 328) reference was made to Fauré-Fremiet's demonstration of the origin of the chitinous membrane of these eggs from their glycogen.

His work was repeated and for the most part confirmed by Szwejkovska. She found 4·85 gm. per cent. (of egg-contents) in the egg at fertilisation, 2·01 after the elimination of the first polar body, and 0·756 after the elimination of the second. Of the 52 per cent. of the glycogen disappearing between the first two points, she found 47 per cent. as glucosamine in the chitinous envelope. Thus the chitin in grams per cent. rose—1·665 per cent. at stage 1, 3·39 per cent. at stage 2, and 3·507 per cent. at stage 3. There was no transformation of carbohydrate into fat, for the fatty acids also diminished in amount during the interval between fertilisation and the first cleavage:

| | Grams % | | |
|---|---|---|---|
| | Volatile | Non-volatile | Total |
| After fertilisation ... ... ... | 0·455 | 0·526 | 0·981 |
| After elimination of second polar body ... | 0·343 | 0·361 | 0·704 |

There was no change in the nitrogen-content.

The silkworm egg has also been the subject of researches on carbohydrate metabolism. The first estimations were those of Vaney & Conte, who noted a steady and gradual diminution of glycogen throughout embryonic life, from 3·08 per cent. dry weight at the time of laying to 0·413 per cent. at the time of hatching. Their figures are shown in Fig. 290 beside the data of Pigorini and of Tichomirov, which correspond very well. There can be little doubt but that the glycogen-content of the whole silkworm egg falls markedly during the post-hibernation period, and it is probable that it rises slightly during hibernation, although it may then remain constant. Before hibernation the glycogen seems to fall a great deal also. Tichomirov pointed out that the formation of chitin in this insect (0·0 to 0·21 per cent.) would probably account for some of the

glycogen disappearing. To these researches we must add that of Kaneko, who noticed histochemically that at hatching no glycogen was present in the silkworm embryo or its egg, a finding quite in agreement with what has already been said.

Rudolfs, using the egg of the tent-caterpillar, *Malacosoma americana*, found a decline from 0·28 per cent. dry weight to 0·15 per cent. during development.

For echinoderm development we have only one complete piece of work. Ephrussi & Rapkine found for *Strongylocentrotus lividus* that the unfertilised eggs had 5·43 per cent. total carbohydrate, the gastrulae 5·46 per cent. and the plutei 3·4 per cent., i.e. at 0, 12 and 40 hours respectively from fertilisation. The corresponding wet weight percentages were 1·36, 1·35 and 0·72. It was therefore clear that during the segmentation stages no carbohydrate disappeared. Utilisation amounting to 50 per cent., however, took place after the stage of gastrulation. The partition between combined and free glucose was also interesting, as follows:

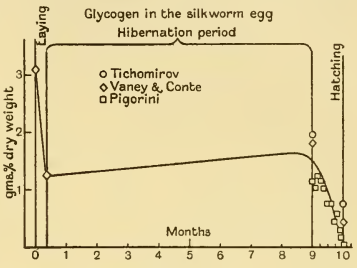



Fig. 290.

| | % of the total carbohydrate | | |
|---|---|---|---|
| Hours ... | 0 | 12 | 40 |
| Non-glycogen and free glucose ... | 4 | 18·3 | 100 |
| Glycogen ... ... ... ... ... | 96 | 81·7 | 0 |

This state of affairs bears a resemblance to that found for the frog's egg by Needham, as mentioned above.

The glycolytic power of the sea-urchin's egg (*Arbacia*) has been studied by Perlzweig & Barron. Measuring the lactic acid formed under various conditions they obtained the following figures:

| | Mgm. lactic acid per gram of egg-protein (experiments of varying duration, mostly 3 hours) |
|---|---|
| Unfertilised control ... ... ... ... | 3·14 |
| Unfertilised plus potassium cyanide ... ... | 5·68 |
| Fertilised control ... ... ... ... | 3·40 |
| 2–8-cell stages control ... ... ... ... | 3·23 |

From this they concluded that lactic acid was formed normally by the developing sea-urchin's egg, and that if its oxidation was inhibited it accumulated as in all other cells. There was a hint of more rapid formation after fertilisation. It will be recalled that Meyerhof in 1911 (see the Section on Respiration) did not succeed in demonstrating any glycogen or free glucose in unfertilised *Strongylocentrotus* eggs, but that Matthews in 1913 (see the Section on Constitution) had found a lipoid in *Arbacia* eggs which contained sugar. In 1927 by improved methods Blanchard did demonstrate the presence of traces of glycogen in *Arbacia* eggs, though not the minutest amount of free glucose. Perlzweig & Barron determined to estimate the total carbohydrate in the eggs before fertilisation, and, using much the same technique as in Needham's studies on the frog, they found about 50 mgm. of glucose per gram of egg protein. It is probable, therefore, that the major part of the carbohydrate in *Arbacia* eggs is present as a mucoid.

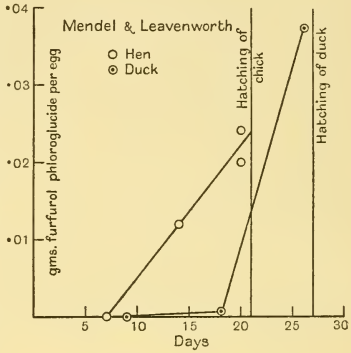



Fig. 291.

As has already been indicated in the Section on Energetics, respiratory quotients closely approaching unity have frequently been obtained during the cleavage stages of echinoderm eggs. Barron has recently been able to fertilise *Arbacia*, *Asterias* and *Nereis* eggs in strictly anaerobic conditions, a finding which suggests carbohydrate metabolism (see p. 758), the cells piling up an oxygen debt. Moreover, direct measurements of glycolysis rate have been made by Ashbel on *Paracentrotus* eggs: revealing an augmentation of N.G.R. on fertilisation. One mgm. of egg nitrogen produced 0·845 mgm. of carbon dioxide per hour from the glucose mixture before fertilisation and 2·32 afterwards. It would be still more interesting to compare this N.G.R. with that of later stages up to the free-swimming pluteus (cf. Section 4·20).

## 8·12. Pentoses

This type of compound has a certain importance in relation to the development of the nucleic acids of the embryo, since Calvery has shown that, besides the ordinary hexose nucleic acid which occurs in animal tissues, the chick embryo possesses also a pentose nucleic acid not unlike that of plants. The only investigation of the pentose-content of an egg is that of Mendel & Leavenworth, who estimated it in hen's and duck's eggs, using Tollens' method. Their figures are shown in Fig. 291, from which it is obvious that the pentose-content of the eggs rises, reflecting the increase in nuclear substance.

## 8·13. Lactic Acid

A good deal more is known about the metabolic behaviour of lactic acid in the hen's egg. It was first found there by Bonnanni in 1914, who regarded it as a normal constituent and reported more in the white than in the yolk. He stated that the fresher the egg the more lactic acid it contained, that there was less in the winter than in the spring, and that by feeding sajodin to hens the lactic acid content of their eggs could be raised. Anno next found traces in the hen's egg-white before incubation, and a rise to the 4th day, but none in the yolk and no rise. Tomita estimated it each day during incubation, and his data are plotted in Fig. 292. An extremely sharp peak is to be seen in both yolk and white on the 5th day of incubation. Tomita drew attention to the coincident marked fall in free glucose, which he knew only through the work of Satô, and expressed the belief that the two phenomena were intimately associated. Perhaps the mechanism by which the lactic acid is transformed into some other compound is itself in course of development, and does not increase in activity as fast as the mechanism which produces lactic

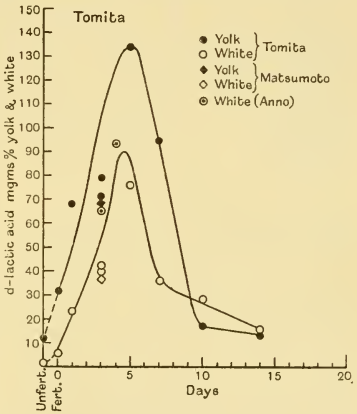



Fig. 292.

acid—this would lead to a temporary accumulation of the intermediate product.[1] Unfortunately we do not yet know the real meaning of this accumulation. In order to penetrate a little farther into the working of these processes, Tomita injected both glucose and alanine into the hen's egg at the beginning of development. He found that the addition of glucose to the egg in this artificial way led to a 50–70 per cent. increase of lactic acid in the white, and to a definite increase in the yolk, though not to more than 15 per cent., and that in only one case. This apparent isolation of the yolk from the events going on in the white during the first week of incubation has been noticed before when we were considering the effect of injections of glucose on the free glucose of the yolk and white. It was interesting that the addition of 200 mgm. of glucose had no more effect in increasing the lactic acid production than the addition of 50 mgm., from which it may be inferred that the relationships considered cannot be simply governed by mass action (see Fig. 293). The lactic acid produced amounts at its maximum to about 46 mgm. per egg, and the amount of free glucose lost during the first 5 days is approximately 55 mgm. (see Fig. 267). When it is remembered that the estimate of the carbohydrate combusted by the 5th day is something very close to 10 mgm., the correspondence is remarkable, and leads to the inference that what is not burned can almost entirely be accounted for as lactic acid. After that we lose sight of it. The decrease in free glucose is more marked in the white than in the yolk (see Fig. 268), but the increase in lactic acid is more marked in the yolk than in the white. Comparative estimations of the lactic acid content of the white yolk and the yellow yolk would be of great interest.

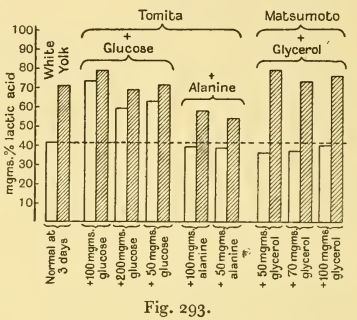



Fig. 293.

[1] It is interesting in this connection that Neuberg, Kobel & Laser have shown the mechanism of lactic acid production in the chick embryo to be identical with that in other tissues. Acetone powders of 8-day embryos give with hexosephosphate good yields of methylglyoxal, showing the presence of active glycolase. The co-enzyme (co-zymase), which assists the ketonaldehydemutase in transforming methylglyoxal into lactic acid, has also been shown to exist abundantly in the embryo rat (Sym, Nilsson & v. Euler), mouse (Waterman), and pig (Kraut & Bumm).

Tomita's injection experiments were continued by Matsumoto several years later, who confirmed some of his normal figures and found that injected glycerol had not the least effect on the magnitude of the lactic acid content in either yolk or white. Tomita himself also studied the effect of autolysis on the lactic acid of the egg. As the diagram in Fig. 294 shows, no increase in the lactic acid of the white was to be seen even after 14 days' autolysis. The addition of glucose or alanine to this had no effect whatever, and no extra lactic acid was formed. The yolk, on the other hand, showed a marked rise in lactic acid when autolysed.[1] As the second experiment demonstrates, it rose for about a week, but later it was found to fall, the lactic acid itself being destroyed. Addition of glucose to the yolk autolysate at the beginning of the experiment led to enormous rises in the lactic acid formed, e.g. from 30 to 300 or 400 mgm., while alanine gave no such increase, whatever its concentration. Tomita concluded from this that the enzyme which hydrolyses the free glucose into the lactic acid existed exclusively in the yolk. For the closely related work of Stepanek, see Section 14·6.

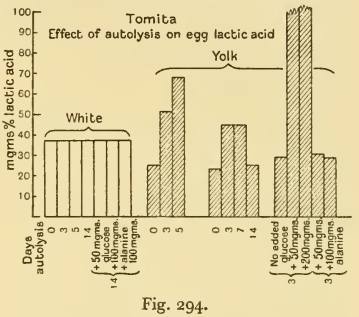



Fig. 294.

Tomita drew attention to the fact that the maximum figure for lactic acid obtained in normal autolysis was quite similar to the maximum observed during normal development.

Ido's experiments were planned rather differently. Taking hen's eggs after variable periods of normal development, he vaselined them so as to exclude air and returned them to the incubator for several weeks. Considerable amounts of lactic acid accumulated, but the correlation between lactic acid formed and glucose destroyed was only precise in the case of embryos at least as old as 5 days. In unincubated eggs thus treated only 27 per cent. of the glucose disappearing could be accounted for by the lactic acid formed. These experiments are of interest in connection with Byerly's findings (see p. 607).

The hen's egg is not the only type which has been examined with

[1] Confirmed subsequently by Needham & Stephenson.

respect to lactic acid. Yoshikawa in 1913 found 3 mgm. per cent. in the white and 12 mgm. per cent. in the yolk of the fresh egg of the marine turtle *Thalassochelys corticata*, and the subject was later pursued by Sendju. It turned out that just as Tomita had found a peak of lactic acid in the development of the bird so Sendju found one in that of the chelonian reptile. Beginning at 8 mgm. per cent. in the fresh egg, the lactic acid rose to 41 per cent. on the 15th day, falling to 15 per cent. on the 45th day. A high value for the newly hatched tortoises may perhaps have been due to the muscular effort involved. The general trend of the figures affords another indication of the similarity between the general metabolic picture of the various sauropsida in pre-natal life.

### 8·14. Fructose

Attention must finally be given to the fructose question which is the most enigmatic aspect of embryonic carbohydrate metabolism. It begins with Claude Bernard, who in 1855 noted that the sugar of the human amniotic liquid was laevorotatory, but that this condition was no longer present at term. In 1904 Gürber & Grunbaum observed that 40 per cent. of the reducing carbohydrate of the amniotic and allantoic fluids of the horse and pig was fructose. This was confirmed and much extended by the classical researches of Paton, Watson & Kerr in 1907, who reported the presence of fructose in the amniotic and allantoic fluids of the sheep, cow, and probably the dog. The blood of the sheep embryo contained in one experiment 420 mgm. per cent. of fructose, but it was not demonstrable in the liver. Then in 1922 Takata found that fructose was the only carbohydrate present in the amniotic fluid of the whale *Balanoptera*, and not long afterwards Orr noted that human and goat foetal blood give the Selivanov reaction, and have an abnormally low rotation. In human foetal blood the fructose/glucose ratio seems to be 1/2 and the former sugar does not completely disappear at birth. There may also be a fructosuria of pregnancy (van Creveld & Ladenius). Are the rare cases of fructosuria in adults cases of arrested development, just as *icterus neonatorum* seems to be a continuation of a normal pre-natal condition?

## SECTION 9

## PROTEIN METABOLISM

### 9·1. The Structure of the Avian Egg-proteins before and after Development

The hen's egg contains at the beginning of development, as we have already seen, about six or seven different proteins and a number of smaller nitrogenous molecules. At the end it consists of another set of proteins, mostly the avian body and serum proteins, a further collection of simpler nitrogenous substances, differently distributed, and, in addition, a quantity of incombustible protein breakdown products, the outcome of the embryonic oxidations. How does the constitution (especially as to amino-acid distribution) of the original egg-protein molecule differ from that of the finished embryo-protein molecule? One answer is afforded by the work of Plimmer & Lowndes who massed together the proteins at 0, 15 and 21 days of incubation, and made van Slyke estimations of the amino-acid distribution, employing Plimmer's modifications of this technique. In Table 130 are shown the results, expressed in percentages of the total nitrogen.

Table 130.

Plimmer and Lowndes' figures (massed proteins):

| | *Experiment* 1 | | | *Experiment* 2 | | |
|---|---|---|---|---|---|---|
| | % of the total nitrogen | | | % of the total nitrogen | | |
| Stage | 0 | 15 days | Hatched | 0 | 15 days | Hatched |
| Amide nitrogen | 8·2 | 8·7 | 8·2 | 9·8 | 9·5 | 8·7 |
| Humin nitrogen | 1·5 | 1·8 | 1·7 | 1·8 | 1·9 | 1·8 |
| Di-amino fraction | | | | | | |
| Total nitrogen | 25·8 | 27·1 | 27·4 | 24·5 | 24·2 | 26·4 |
| Amino nitrogen | 14·8 | 15·0 | 14·5 | 13·2 | 11·3 | 12·2 |
| Non-amino nitrogen | 11·0 | 11·9 | 13·0 | 11·3 | 12·9 | 14·1 |
| Arginine nitrogen | 14·3 | 15·0 | 16·8 | 13·2 | 13·5 | 14·5 |
| Histidine nitrogen | 0·4 | 1·2 | 0·5 | 2·1 | 4·8 | 4·9 |
| Lysine nitrogen | 11·0 | 10·9 | 10·2 | 9·2 | 6·4 | 6·6 |
| Cystine nitrogen | 1·8 | 1·7 | 2·6 | 1·2 | 1·2 | 0·6 |
| Mono-amino fraction | | | | | | |
| Total nitrogen | 64·0 | 61·3 | 60·2 | 63·7 | 62·9 | 62·8 |
| Amino nitrogen | 67·1 | 64·7 | 59·1 | 59·3 | 57·1 | 55·3 |
| Non-amino nitrogen | — | — | 1·1 | 4·3 | 5·8 | 7·4 |
| Arginine nitrogen | 4·4 | 3·6 | 3·2 | 4·0 | 3·1 | 3·3 |
| Cystine nitrogen | 3·5 | 4·9 | 5·7 | 3·9 | — | 3·3 |

Over the whole period the percentage of amide and humin nitrogen showed no change, except a very slight decrease in amide nitrogen in one experiment. The total di-amino nitrogen percentage, however, increased to the extent of 2 per cent., and, corresponding with this, the arginine nitrogen increased by 1 per cent. Histidine and lysine probably account for the difference. Conversely the mono-amino-acids decreased—from 64·0 to 60·2 in one experiment, and from 63·7 to 62·8 in another. The non-amino nitrogen of this fraction, on the other hand, increased, and, as far as could be ascertained with these methods, the cystine nitrogen did so too. As the figures for bromine absorption decreased continually through development, Plimmer & Lowndes suggested that the cystine was increasing relatively at the expense of tyrosine, but, as Plimmer & Phillips had previously shown, the bromination method is not very quantitative, nor even very specific. The main result of the investigation was the finding that the mono-amino-acids decreased and the di-amino-acids increased, with the suggestion that the former were those principally used for furnishing energy by combustion. As will be mentioned later, an analogous process seems to go on in the eggs of the trout and the salamander, the only other material which has been treated from this point of view.

Very similar experiments to those of Plimmer & Lowndes were carried out by Russo, whose main interest lay in the origin of the purine bases. He had already found a diminution in the arginine and histidine content of the echinoderm testis corresponding to an increase in the purine bases, and, in accordance with the original suggestion of Hopkins & Ackroyd, he was inclined to regard that way of derivation as universal. Russo's results with avian eggs are shown in Table 131; they were obtained with the van Slyke method applied to the massed proteins of all the parts of the egg-contents. It is evident from the figures that in his experiments the amide nitrogen remained constant (agreeing thus with those of Plimmer & Lowndes), as also did the cystine (differing from theirs). Russo's treatment of the cystine question, however, cannot be considered final, for, as Plimmer & Lowndes have shown in another paper, some of the cystine passes into the filtrate from the phosphotungstates of the hexone bases in the van Slyke method, and must be looked for there. The question is also complicated by the presence of Mueller's sulphur-containing amino-acid in the mono-

amino-acid fraction. Perhaps more serious, therefore, is the divergence between the results of Russo and those of Plimmer & Lowndes over the acids arginine and histidine, for, whereas in the latter they definitely though slightly increased, in the former they equally definitely decreased. And the mono-amino-acids, which in Plimmer & Lowndes' work decreased, in Russo's work increased, an effect seen in both the amino and non-amino fractions. All the changes in Russo's experiments were greater relatively than those in Plimmer & Lowndes'.

Table 131.

Russo's figures (massed proteins):

| Days ... | 0 | 10 | 15 |
|---|---|---|---|
| Amide nitrogen ... ... ... | 5·12 | 5·17 | 5·15 |
| Humin nitrogen (not determined) | | | |
| Di-amino fraction | | | |
| Total nitrogen... ... ... | 24·62 | 24·26 | 20·80 |
| Arginine nitrogen ... ... | 11·84 | 10·25 | 7·89 |
| Histidine nitrogen ... ... | 3·68 | 4·04 | 3·13 |
| Lysine nitrogen ... ... | 9·10 | 9·97 | 9·78 |
| Cystine nitrogen ... ... | 0·49 | 0·47 | 0·47 |
| Mono-amino fraction | | | |
| Total nitrogen ... ... | 48·25 | 51·90 | 52·91 |
| Amino nitrogen ... ... | 40·79 | 42·35 | 42·61 |
| Non-amino nitrogen ... ... | 7·46 | 9·55 | 10·30 |
| Nitrogen unaccounted for ... | 21·52 | 18·20 | 20·67 |

Calvery's figures (massed proteins, including shell):

| | % of the total nitrogen | | | | |
|---|---|---|---|---|---|
| Days ... | 0 | 5 | 10 | 15 | 20 |
| Acid melanin nitrogen ... | 0·88 | 0·81 | 1·04 | 0·94 | 0·99 |
| Amide nitrogen ... ... | 8·69 | 6·84 | 8·44 | 8·88 | 8·22 |
| Humin nitrogen ... ... | 0·90 | 0·89 | 1·15 | 1·49 | 1·65 |
| Di-amino fraction | | | | | |
| Total nitrogen ... ... | 26·30 | 27·75 | 27·05 | 25·93 | 28·25 |
| Arginine nitrogen ... | 14·53 | 14·72 | 13·25 | 13·32 | 15·82 |
| Histidine nitrogen ... | 1·82 | 3·31 | 4·74 | 3·01 | 1·69 |
| Lysine nitrogen ... ... | 8·93 | 8·65 | 8·53 | 9·60 | 10·12 |
| Cystine nitrogen... ... | 0·80 | 0·91 | 0·72 | 0·93 | 0·65 |
| Mono-amino fraction | | | | | |
| Total nitrogen ... ... | 64·05 | 62·54 | 62·25 | 63·95 | 62·00 |
| Amino nitrogen ... ... | 61·40 | 60·35 | 58·72 | 59·40 | 55·82 |
| Non-amino nitrogen ... | 2·65 | 2·19 | 3·53 | 4·55 | 6·18 |

The third investigation along these lines was that of Calvery, who reversed Russo's findings and agreed with Plimmer & Lowndes that the mono-amino-acids were combusted rather than the di-amino-

acids. As Table 131 shows, Calvery found no change in acid melanin, amide, or cystine fractions, a fall followed by a rise in the arginine fraction and a rise in the lysine fraction. His data, however, differed from those of previous workers by including the shells, so the subject is by no means closed and in spite of the large amount of laborious work that has been done on it we cannot as yet be said to know much about the origin of either the nucleins or the incombustible end products. The change over from amino to non-amino nitrogen in the filtrate from the bases, which appears in all three sets of figures may be due to the accumulation of proline and oxyproline.

Table 132.

Sznerovna's figures:

| Days | Proteins of | % of the total nitrogen: Ammonia nitrogen | Melanin nitrogen | Di-amino nitrogen | Mono-amino nitrogen |
|---|---|---|---|---|---|
| 0 | White ... ... ... | 6·64 | 2·18 | 64·52 | 26·66 |
| | Yolk ... ... ... | 7·88 | 0·73 | 66·02 | 25·37 |
| | White and yolk massed | 7·14 | 1·60 | 65·12 | 26·14 |
| 14 | Embryo ... ... | 5·58 | 3·02 | 58·15 | 33·25 |
| 18 | Embryo ... ... | 5·98 | 2·46 | 64·07 | 27·49 |
| | White and yolk massed | 7·14 | 1·45 | 64·77 | 26·64 |

Calvery's figures for whole egg (including shell):

| Days | Histidine nitrogen % of total nitrogen: van Slyke distribution method | van Slyke total nitrogen in histidine fraction | Bromination Plimmer & Phillips | Colorimetric Hanke & Koessler | Mercuric sulphate Kossel & Patten | Isolation as flavianate |
|---|---|---|---|---|---|---|
| 0 | 2·31 | 5·86 | 6·70 | 5·03 | 3·75 | 3·81 |
| 5 | 1·36 | 6·07 | 5·83 | 4·93 | 3·76 | 3·68 |
| 10 | 4·74 | 6·14 | 5·90 | 4·42 | 3·79 | 3·51 |
| 15 | 3·01 | 6·25 | 6·00 | 4·00 | 3·13 | 3·13 |
| 20 | 1·69 | 7·98 | 6·01 | 3·41 | 1·35 | 1·38 |

| Days | Arginine and lysine nitrogen % of total nitrogen: Arginine nitrogen: van Slyke distribution method | Arginine nitrogen: Total nitrogen in arginine fraction | Arginine nitrogen: Alkaline hydrolysis van Slyke | Arginine nitrogen: Isolation as flavianate | Lysine nitrogen: van Slyke distribution method | Lysine nitrogen: Total nitrogen in lysine fraction | Lysine nitrogen: Isolation as picrate |
|---|---|---|---|---|---|---|---|
| 0 | 14·63 | 11·04 | 9·90 | 9·48 | 8·40 | 10·90 | 7·04 |
| 5 | 14·74 | 9·93 | 9·86 | 9·63 | 11·00 | 8·86 | 6·40 |
| 10 | 13·25 | 11·63 | 9·58 | 9·59 | 8·53 | 8·60 | 5·78 |
| 15 | 13·32 | 12·66 | 9·33 | 9·53 | 9·60 | 9·10 | 5·05 |
| 20 | 15·82 | 12·84 | 9·91 | 9·83 | 10·12 | 8·30 | 4·06 |

Experiments of a somewhat similar kind were made by Sznerovna, who took the proteins of yolk, white and embryo at different stages, and hydrolysed them, effecting then a simple separation into di-amino and mono-amino fractions. Her figures are given in Table 132. They do not give any answer to the problem attacked by Plimmer & Lowndes and by Russo, for she did not state the results for the massed embryo and yolk proteins at the end of incubation, and they cannot be calculated in the absence of information concerning the relative weights of her 18th day embryos. Nevertheless her data for the amino-acid distribution in the massed white and yolk proteins strongly support the view that there is no intrinsic change in them during development, for it is substantially the same on the 18th day as at zero hour. On the other hand, her figures show a big difference between the embryo protein of the 14th and that of the 18th day, trending in a direction favourable to Plimmer & Lowndes rather than to Russo. The mono-amino-acids in the embryo protein certainly seem to be diminishing, and the di-amino-acids to be increasing, although such a change in the composition of the body protein contradicts Cahn's suggestion of its constancy. The whole question is confused, and needs further analysis than it has received.

## 9·2. Metabolism of Individual Amino-acids

Individual amino-acids, also, have been estimated in the hydrolysates of the massed egg-proteins at different stages of development. Thus Abderhalden & Kempe in 1907 obtained the amounts of amino-acids in the egg at 0, 10 and 21 days, tyrosine by simple crystallisation, glutamic acid by the hydrochloric acid method, and glycine by esterification. In grams per cent. of the egg, none of the three manifested any change, but the methods employed were not delicate enough to settle the question. Levene was another pioneer along these lines, but his data were few, and so erratic that no good purpose would be served by discussing them.

An important series of experiments on the metabolic behaviour of individual amino-acids during incubation was carried out by Sendju. Tryptophane in the embryo, the yolk and the white was estimated by the von Fürth colorimetric technique. As Fig. 295 shows, there was a marked diminution of it in absolute amount throughout the period, and this diminution took place in two stages separated from each other by a plateau. Sendju identified the first of the two falls

with the production of haemoglobin and the second with the formation of the bile pigment, which is so noticeable a constituent of the egg in the last few days before hatching. Sendju estimated tyrosine in the same way, using the method of von Fürth & Fleischmann, and the results he obtained are plotted in Fig. 296. The general picture is very like that for tryptophane—the total amount of the amino-acid decreases, both in yolk and white, while the fraction of it contained in the embryonic body rises to meet the descending total curve at hatching. Sendju's values for the total amount of tyrosine agree to

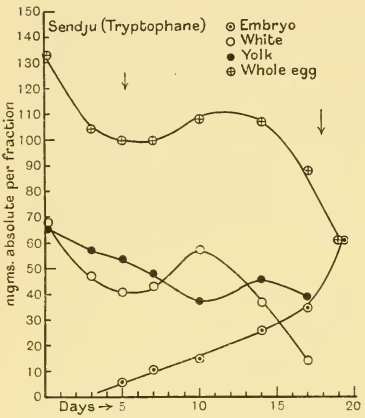



Fig. 295.

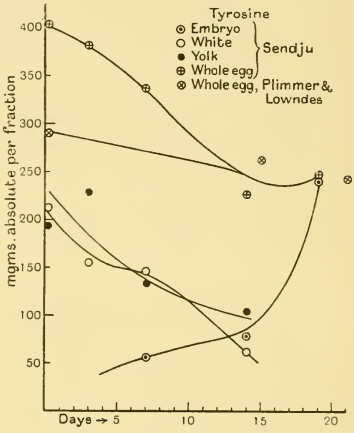



Fig. 296.

some extent with those given by Plimmer & Lowndes, better at the latter part of the curve than at the former. The fall was regarded by Sendju as indicating the utilisation of the tyrosine for hormones and other special purposes.

Sendju's estimations of the content of arginine, histidine and lysine are shown in Figs. 297 and 298. There is a general similarity between the graphs, for the amount of substance in the embryo regularly rises till it has absorbed all that outside, while the yolk and white curves show that the material of the white is used regularly before that of the yolk. The behaviour of the curves for total amino-acid, however, shows some variation, histidine distinctly rising, lysine less so, and

arginine distinctly falling. Sendju himself attached no special importance to these variations, which would agree with those found by Plimmer & Lowndes in the case of histidine and lysine, but not in that of arginine.

The best work on this subject is that of Calvery who made a comparative study of histidine, arginine and lysine, using a variety of different methods.[1] His figures, which are given in Table 132, show, as regards the histidine (percentage of total nitrogen of whole egg,

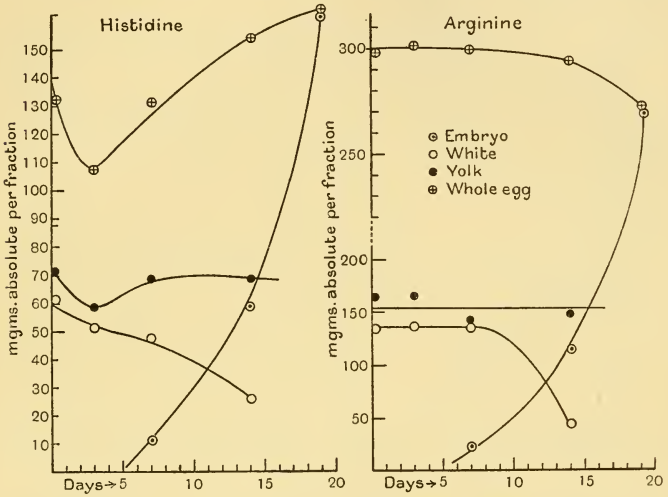



Fig. 297.

shell included), in one case no change, in another case a slight increase, and in four cases, a definite decrease. Calvery regards Sendju's increase as due to errors of technique, but thinks Russo's decrease was genuine. As regards arginine, all four methods showed no change. Sendju's diminution is again considered by Calvery as being due simply to analytical errors, but no explanation is available for the large fall found by Russo. Out of three methods used for lysine, one showed no change and two a decrease, which Calvery thought

[1] Calvery's later work confirmed Sendju's fall in tyrosine and cystine but showed no fall in tryptophane.

probably real. This again is not in agreement with the work of Plimmer & Lowndes; Sendju, and Russo.

The fact of the matter is that the estimation methods available are insufficiently delicate to show up clearly the slight changes which occur in these amino-acid distributions. One reflection, at any rate, may be made, namely that the raw material of the embryo is not very different from the embryo itself: half the di-amino-acids, for instance, do not have to be made into mono-amino-acids. Would

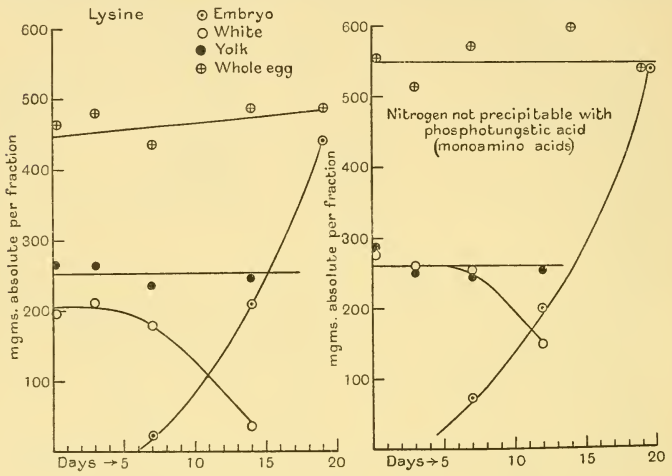



Fig. 298.

this be equally true of an embryo which did not have to suppress its protein catabolism, as is the case with terrestrial animals? Parallel studies with aquatic eggs should certainly be undertaken. And one also wonders, if the hen's egg economises its amino-acids to such an extent, whether they ever go down to individual simplicity at all, or whether they do not rather enter the embryo in the form of proteose bundles.

Sendju did not give his amino-acid results in terms of the weight of the embryo, but they have been calculated in this way, and are shown in Fig. 299. Sagara has also made some estimations of the arginine, histidine and lysine content of the embryo, but they

were few in number, and the values were extremely small, disagreeing with the results of all other workers. They have not therefore been included in these graphs. Fig. 299 illustrates, of course, the increasing dryness of the embryo. On the same graph are plotted the more recent results of Cahn, who has estimated the arginine contained in the embryo during incubation. These are seen to agree to some extent with those of the Japanese worker, the difference being probably due to the fact that Cahn only gives his dry weights of embryos after removal of fat. These curves, however, do not bring out any important relation. The value of Cahn's work lies rather in the fact that, just as Plimmer & Lowndes examined the structure of the protein molecule of the whole egg throughout incubation, so Cahn examined that of the embryo. As Table 133 demonstrates, the percentage of nitrogen remains perfectly constant in the dry fat-free and ash-free protein of the embryonic body. A few figures were also given by Cahn showing a constant nitrogen-content of the proteins of the yolk and white. This would suggest that, although the proteins of the egg as a whole undergo a rearrangement in being converted from egg into embryo proteins, the latter do not themselves change through embryonic life, but remain of the same constitution when once they are formed. Such a conclusion is strongly supported by Cahn's figures for the arginine-content of the protein of the embryonic body, for, as Table 133 shows, this also is constant. The very slight variations in the nitrogen-content of the protein molecule were regarded by Cahn either as technical errors due to incomplete removal of fat and ash, or perhaps as being due to the presence in different organs of different proteins varying slightly in this respect, and appearing now in one predominance, now in another, according to the differential growth of the various parts of

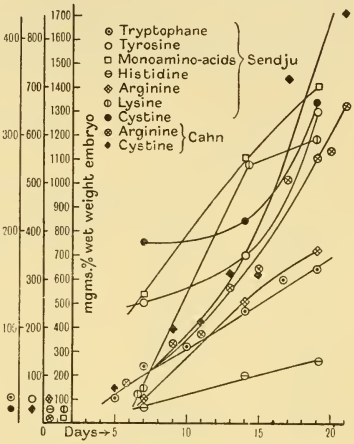



Fig. 299.

the body. The arginine referred to in Fig. 299 and Table 133 was exclusively that resulting from the hydrolysis of the proteins, and was estimated by decomposition with arginase and estimation of the urea so formed by the xanthydrol reagent. Cahn also investigated the amount of total arginine in the whole egg during incubation and found no change whatever. This was contrary to Sendju's findings, but agreed more or less with those of Plimmer & Lowndes. Cahn stated that the arginine-content of the proteins of the non-embryonic parts of the egg remained approximately constant (7 per cent.) throughout incubation. These observations certainly make it appear as if neither the egg proteins nor the embryo proteins change intrinsically during development, apart from the breakdown which has to go on in order that the one may be transformed into the other.

Table 133.

Cahn's figures:

| Days | Nitrogen % of proteins water-fat- and ash-free: Embryo | Nitrogen % of proteins water-fat- and ash-free: Yolk and white | Arginine % of total protein nitrogen in embryo | Arginine % of water-, fat- and ash-free embryonic proteins |
|---|---|---|---|---|
| 5 | 15·85 | — | 46·0 | — |
| 6 | 16·1 | — | — | 7·16 |
| 8 | 15·55 | — | 47·0 | 6·8 |
| 9 | 15·75 | 15·00 | 46·2 | 7·0 |
| 11 | 15·67 | — | 47·3 | 7·16 |
| 13 | 15·55 | — | 46·3 | 7·2 |
| 15 | 15·50 | 14·70 | 48·0 | 7·1 |
| 17 | 15·35 | — | — | — |
| 18 | — | 14·50 | — | — |
| 19 | 15·55 | — | 46·0 | — |
| 21 | 15·50 | 15·55 | 45·6 | 7·10 |

Another amino-acid which has been closely studied is cystine. Sendju's experiments, in which all the cystine, both free and combined in proteins, was estimated by the Okuda iodimetric method, did not give very illuminating results, though the passage of this amino-acid from the yolk and the white into the embryo is easily seen in Fig. 300. The diminution in total cystine stands in contradiction with the findings of Plimmer & Lowndes. Expressed in milligrams per cent. wet weight, the cystine as measured by Sendju can be observed in Fig. 299. The analogous curve constructed from Cahn's data is shown on the same graph, but his more important finding was that the amount of cystine in the protein molecule was not constant. The figures in Table 134 illustrate this. For the middle part

of development the percentage of cystine in the embryonic protein is fairly steady, but during the first and last days it rises. The rise at the end of the period might be due to the appearance of feathers and the cystine of their keratin, but, as Table 108 shows, their weight is small, and will probably not account for it. Cahn's explanation involved the postulate of a central fixed nucleus in the embryonic protein molecule, of which arginine would be a constituent member, while cystine would not. These are hypotheses on which much further work might be profitably carried on.

According to Calvery, the cystine/cysteine ratio of the hen's egg falls from 5·9 to 0·8 during development.

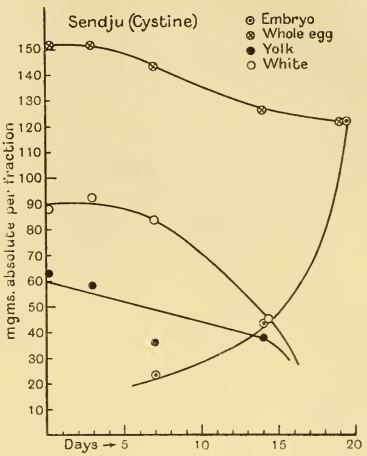



Fig. 300.

Table 134.

Cahn's figures:

| Days | Cystine grams % of protein in embryonic body | Cystine grams % of protein nitrogen in embryonic body |
|---|---|---|
| 5 | 3·61 | 22·8 |
| 8 | 4·25 | 27·0 |
| 9 | 4·37 | 28·2 |
| 11 | 4·38 | 27·0 |
| 13 | 4·87 | 24·6 |
| 15 | 4·20 | 27·1 |
| 17 | 5·53 | 35·9 |
| 21 | 5·03 | 32·6 |

## 9·3. The Relations between Protein and Non-protein Nitrogen

Many investigators, wishing to unravel the processes by which the egg proteins are transformed into those of the embryonic body, have estimated the non-protein and free amino-acid nitrogen in various

parts of the egg from time to time, and it will be convenient at this point to consider what knowledge their work has led to. Albumoses and peptones have been shown to be present in the egg-white by qualitative analysis on the 15th day by Fischel and on the 6th by Emrys-Roberts. One of the first of these investigators was Tomita, who removed the proteins from the white and the yolk by boiling with

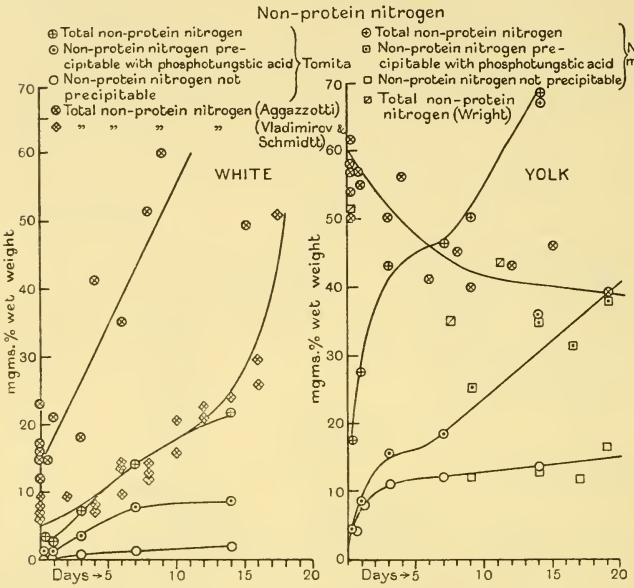



Fig. 301.

acetic and precipitating with tannic acid, after which he estimated the total non-protein nitrogen in the filtrates, the nitrogen precipitable by phosphotungstic acid (the di-amino-acids, peptides, and some of the cystine), and, finally, that not so precipitable (the mono-amino-acids, and some of the cystine and arginine). His results are shown in Fig. 301, together with those of Nakamura, who later confirmed him. Unfortunately neither of them took into account the varying water-content of the white and the yolk, so that the rise found in

their values cannot be assessed at its true value from their data alone. As the white is drying up, and the yolk becoming wetter during the first 10 days of incubation, Tomita's figures might be said to be unduly small for the yolk and rather too large for the white, a correction which would exaggerate the difference between them.

Later still, another Japanese worker, Takahashi, estimated the free bases in the whole egg during development. All were found to rise:

| | Milligrams per whole egg | | | |
|---|---|---|---|---|
| Days | Purine nitrogen | Histidine nitrogen | Arginine nitrogen | Lysine nitrogen |
| 0 | 0·04 | 0·13 | 1·02 | 6·55 |
| 5 | — | 0·12 | 1·90 | 6·97 |
| 9 | 0·04 | 0·02 | 2·31 | 9·38 |
| 14 | 0·38 | 0·19 | 3·40 | 15·97 |
| 17 | 1·69 | 0·20 | 4·03 | 16·30 |
| 19 | 0·95 | 0·38 | 5·15 | 27·35 |

The general order of magnitude of these figures agrees well with that shown in Fig. 301.

Aggazzotti, who was working at much the same time as Tomita, took the changing water-content of the yolk and white into consideration. Aggazzotti precipitated the proteins in the yolk and white by the Costantino acid sublimate technique, estimated the total and the amino nitrogen in the filtrate, and then, hydrolysing the proteins, estimated their total nitrogen, their amino nitrogen and their ammonia. The results he obtained are shown in Figs. 302 and 303. As regards the white, there was no change at all in the total protein nitrogen, or in the bound amino-acid nitrogen, though the ammonia of the proteins diminished by more than 50 per cent. The significance of this fall in the protein ammonia is not at all clear. Matters took very much the same course in the yolk, where the protein nitrogen and the bound amino-acid nitrogen remained constant, while the bound ammonia rose a little before falling. The free amino nitrogen, plotted in Fig. 304, showed practically no change when related to dry weight in the yolk, but rose and fell in the white with a maximum at the 8th day. However, when the free amino nitrogen was compared with the total free nitrogen, so that a measure was obtained of the extent to which the free amino-acids accounted for the non-protein nitrogen at any moment, the curves of Fig. 305 were obtained. From them it appears that there is a maximum of free

amino-acids (expressed in this way) in the yolk at about the 3rd day and in the white at about the 7th day. As will appear presently, this result fits in well enough with other data obtained on the embryo itself. As regards the wet weight of the white, Aggazzotti was in agreement with Tomita in finding an increase of free amino-acids so related, in the case of the yolk precisely the opposite held good.

The main conclusion which can be drawn from the work of Tomita and Aggazzotti is that the changes in amino-acid concentration in the yolk and white are rather minute. There was certainly no *a priori* reason for expecting big changes, for the transfer of amino-acid mole-

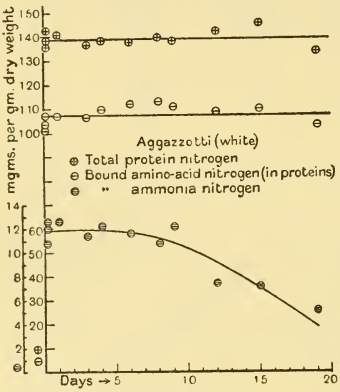



Fig. 302.

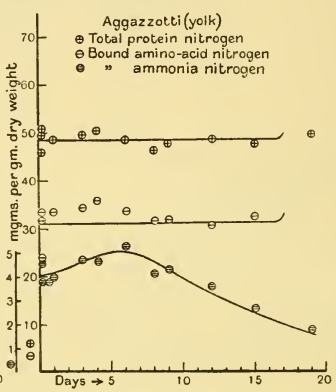



Fig. 303.

cules from egg to embryo might vary very greatly in intensity without involving a difference in absolute amount or concentration of the intermediary substances.

Later, Vladimirov & Schmidtt precipitated the proteins of the egg-white during development by the Fischer-Bang uranium acetate method, and by doing Kjeldahl estimations on the precipitate and the filtrate obtained figures for the protein and non-protein nitrogen. The former increased steadily as the water-content of the white diminished; the latter also increased steadily, so that the ratio protein nitrogen/non-protein nitrogen was constant. Both are plotted beside Tomita's results in Fig. 301, with which they agree. Aggazzotti's results are much higher all through. Thus all observers agree

that the non-protein nitrogen increases per cent. wet weight in the white, but for the yolk Tomita's rising curve stands in contrast with Aggazzotti's falling one. A private communication from Wright states that recently results have been obtained confirming Aggazzotti on this point (see Fig. 301). Free amino-acids have been found by Fiske & Boyden in the allantoic liquid of the chick, to the extent of 13 mgm. per cent. on the 5th and 7 mgm. per cent. on the 10th day of incubation. This is lower than the amino nitrogen of the embryonic blood which Vladimirov & Schmidtt found to vary somewhat erratically between the 13th day and hatching around an average of 54·2 mgm.

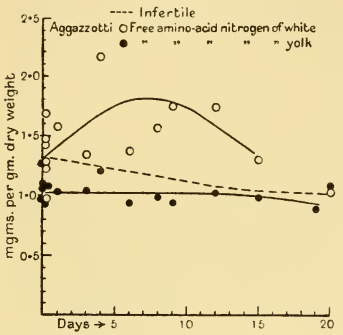



Fig. 304.

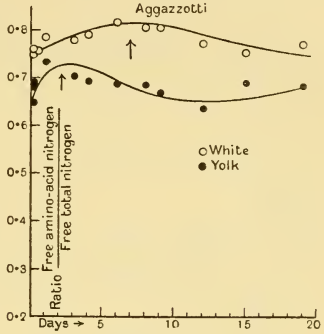



Fig. 305.

per cent. After hatching it has an average value of 52·7 mgm. per cent. Such high values are due to the nucleated erythrocytes.

Certain Japanese workers filled an obvious gap in the data which have so far been reviewed by estimating the total nitrogen of the entire egg throughout, and the whole of the free or combined amino-acid nitrogen. The figures of Sakuragi and of Idzumi are shown in Table 135. As Liebermann had originally thought probable, and as Tangl & von Mituch had later made very likely, there is no change at all in the total nitrogen of the whole egg. Not more than an infinitesimal quantity of this element can escape, in agreement with Krogh's findings discussed in the Section on respiration. Sakuragi, as the table shows, estimated the nitrogen in the proteins coagulable with acetic acid, on the one hand, and the

## Table 135.

(All values in milligrams nitrogen per 100 gm. egg-contents.)

| Day | Total nitrogen of all egg-contents, embryo, yolk white, and membranes: Sakuragi | Sznerovna | Idzumi | Whole egg: Sakuragi: Nitrogen coagulable with acetic acid | Sakuragi: Nitrogen not so coagulable | Needham: Nitrogen coagulable with trichloracetic | Needham: Nitrogen not so coagulable | Idzumi: Non-protein nitrogen | Idzumi: Free amino-acid nitrogen | Yolk: Iljin: Total nitrogen | Iljin: Protein nitrogen | Iljin: Non-protein nitrogen | Distribution of total nitrogen of whole egg: Sznerovna: White | Sznerovna: Yolk | Sznerovna: Embryo | Tangl & von Mituch: White | Tangl & von Mituch: Yolk | Tangl & von Mituch: Embryo |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 2055 | 1970 | 2024 | 1846 | 209 | 1960 | 90 | 19·9 | — | 900 | 895 | 5 | 1180 | 793 | — | | 1860 | — |
| 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 3 | — | — | 1956 | — | — | — | — | 19·4 | — | — | — | — | — | — | — | — | — | — |
| 4 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 5 | — | — | — | — | 203 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 6 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 7 | — | — | 1993 | — | — | — | — | — | 19·05 | — | — | — | — | — | — | — | — | — |
| 8 | 1982 | — | — | 1785 | 197 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 9 | — | — | 2032 | — | — | — | — | 56·9 | 23·6 | — | — | — | — | — | — | — | — | — |
| 10 | 2025 | — | 2120 | 1818 | 207 | — | — | 71·7 | — | — | — | — | — | — | — | — | — | — |
| 11 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 12 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 13 | 2040 | — | — | 1818 | 222 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 14 | — | — | 1990 | — | — | — | — | 79·6 | 34·5 | — | — | — | — | — | — | — | — | — |
| 15 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 16 | 1956 | — | 2178 | 1703 | 252 | — | — | 90·4 | — | — | — | — | — | — | — | — | — | — |
| 17 | 2050 | — | — | 1813 | 237 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 18 | — | — | 1924 | — | — | — | — | 133·1 | 41·6 | — | — | — | 1170 | — | 855 | — | — | — |
| 19 | — | — | — | — | — | — | — | 152·2 | — | — | — | — | — | — | — | — | — | — |
| 20 | 2070 | 2030 | 1982 | 1698 | 377 | — | — | — | 35·3 | 770 | 574 | 196 | — | — | — | — | 520 | 1340 |
| 21 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Av. | 2025 | 2000 | 2021 | 2055 | | 2055 | | | | | | | | | | | | |

nitrogen in the proteins not so coagulable plus that of all other nitrogenous compounds, on the other. Owing to protein combustion, the former diminishes and the latter rises. Idzumi, on the contrary, obtained the non-protein nitrogen separately from the uncoagulable protein nitrogen, and the free amino nitrogen in addition to that. The free amino nitrogen remains almost constant during development, thus agreeing with the data already presented, but the total non-protein nitrogen rises owing to the accumulation of protein waste products.

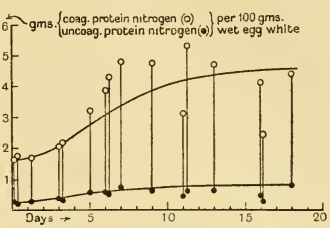


Fig. 306.

The protein just referred to, which is not coagulable with acetic acid, is, of course, ovomucoid, and in the Section on carbohydrate metabolism an account has been given of our knowledge of the physiology of this compound. Trichloracetic acid was found by Hiller & van Slyke to make a sharp separation between proteins of all kinds and nitrogenous bodies of lower molecular weight. Sakuragi gave for zero hour of development the values of 1846 mgm. per cent. of coagulable protein nitrogen (acetic acid) and 209 mgm. per cent. not so coagulable. Using trichloracetic acid, Needham found 1960 mgm. per cent. for all protein nitrogen and 90 mgm. per cent. non-protein nitrogen. The increase in the protein nitrogen caught by the trichloracetic acid method over that caught by the simple boiling with acetic acid was thus 114 mgm. per cent. Komori found by alcohol precipitation 480 mgm. of ovomucoid per egg, i.e. 124 mgm. per cent. of ovomucoid nitrogen, which gives close agreement with the extra 114 found on using trichloracetic acid as the precipitant.

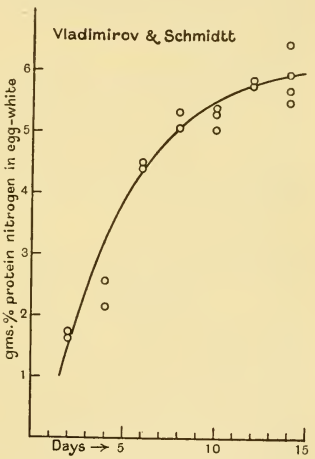


Fig. 307.

The subsequent fate of the ovomucoid fraction and the part played by it in metabolism have been discussed in Section 8. Here, however, it may be noted that the ovomucoid is not absorbed by the embryonic vessels at a different rate from the ovoalbumen. This was demonstrated by Bywaters' experiments, a graph of which is given in Fig. 306. The ratio of the coagulable to the uncoagulable protein nitrogen remains the same throughout development, though in some cases Bywaters' points are rather erratic, and there may well be some interesting relations hidden under this approximation (see also Fig. 307).

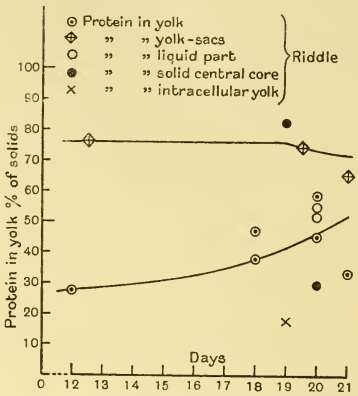



Fig. 308.

Other points also emerge from Table 135. Sznerovna's figures illustrate the transfer of nitrogen from yolk and white to embryo, the embryo having at the end about as much nitrogen as the yolk had at the beginning. Iljin's figures illustrate the large increase (perhaps due to ovomucoid) in the unprecipitable nitrogen of the yolk towards the end of development. But more illuminating are the figures of Riddle represented graphically in Fig. 308, for they show that, in per cent. of the dry solids in the yolk, the yolk-proteins rise during the last 5 or 6 days of incubation. This is due to a preference on the part of the embryo for lipoids and fats, the concentration of which correspondingly rapidly decreases, and the graph has only to be compared with that in Fig. 252, where the absorption intensity of the embryo at different times is given, to show how good the correlation is. For 5 or 6 days before hatching

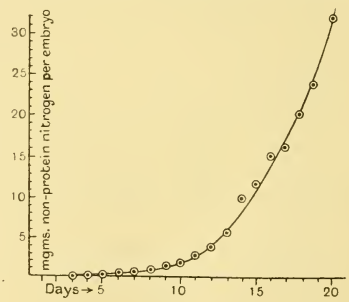



Fig. 309.

the absorption intensity for protein is falling rapidly and that for fatty substances is rising rapidly. On the other hand, it is known that the contents of the albumen-sac tend to be included in the yolk at the end of development, about the time of the opening of the sero-amniotic duct, and this may partly explain Riddle's results. Riddle also investigated the protein content of the yolk-sac itself from the 12th day onwards, finding practically no change, and obtained various figures for the solid central core of yolk, the more liquid part, and the yolk enclosed in the walls of the sac. These are given on the graph, but, as they are somewhat erratic and depend on an arbitrary selection of the material, they are not so important as the rest. These viscous bodies in the central core, which differed very markedly in chemical composition, were explained by Riddle as being in one case unaltered yolk from the beginning of development, and in the other case a clump of egg-white driven into the yolk and imperfectly infiltrated with it. Riddle also found that, when yolk is being resorbed in the hen by the follicle which secreted it, there is a more rapid removal of lipoids than of fats, and of fats than of proteins.

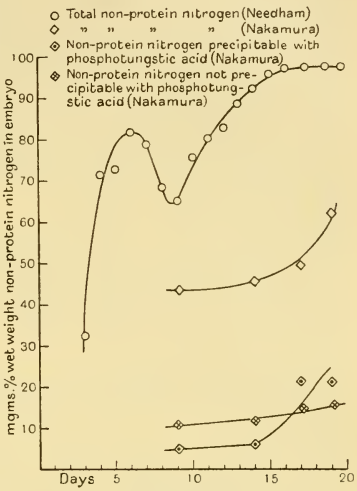



Fig. 310.

It will have been remarked that, so far, nothing has been said about the non-protein nitrogen within the embryo. The early work of Demant on human and guinea-pig foetuses, in which a high concentration of peptones and albumoses was found, was discredited by Neumeister. In 1927 Needham estimated the nitrogen in the trichloracetic filtrate by Rose's modification of the Kjeldahl method. The curve obtained was regular (Fig. 309), but more interesting points were brought out by the expression of the non-protein nitrogen of the embryo in terms of wet weight. Fig. 310 shows this, together

with a few later figures of Nakamura, which are not concordant. Concentrating attention on Fig. 311 for the moment, it can be seen that the wet weight curve, after a peak on the 6th day and a depression on the 9th, gains a plateau and remains there for the rest of development. The dry weight curve presents the same peak and the same depression, but, since the embryo is growing much drier as it increases in size, the dry weight curve drops away steadily after the 10th day. It is significant that there is a very vigorous period of protein absorption[1] before the 5th day, and another from the 14th to the 18th days. The depression in the curves of Fig. 311 comes just between the two maxima of protein absorption. The amount of non-protein nitrogen in 100 gm. of embryo is related, then, to the amount of protein nitrogen which 100 gm. of embryo is receiving from the rest of the egg. Reference to Fig. 250 illustrates this.

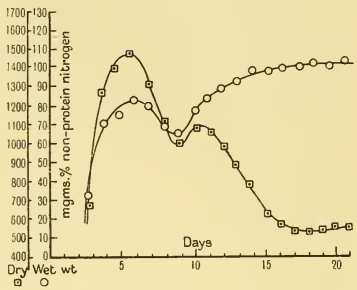



Fig. 311.

Another curve which is worth studying is the curve for non-protein nitrogen in percentage of the total nitrogen (Table 136 and Fig. 312). Low at first, the value rises to attain a peak on the 6th day, and thereafter falls, except for a slight oscillation about the 9th day, probably comparable in cause with those noted as occurring at the same time in the curves for non-protein nitrogen as percentages of wet and dry weight. It may be said, then, that, though little change seems to take place in the non-protein nitrogen outside the embryo, yet, inside it, definite peaks are found, and that these correspond with the periods of greatest intensity of protein absorption, such

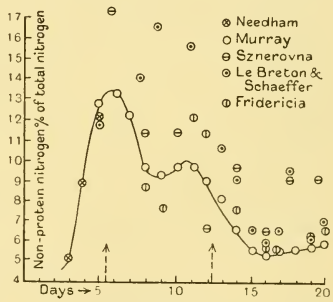



Fig. 312.

[1] This does not, of course, mean absorption of intact protein; see pp. 920 ff.

## Table 136.

| Day | Total nitrogen in milligrams per embryo: Murray | Fridericia | LeBreton & Schaeffer | Sznerovna and Needham | Non-protein nitrogen Needham | Water-soluble non-protein nitrogen in % of total nitrogen in embryo: Murray | Fridericia | LeBreton & Schaeffer | Sznerovna and Needham |
|---|---|---|---|---|---|---|---|---|---|
| 0 | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — | — | — | — |
| 3 | — | — | — | 0·177* | 0·0095 | — | — | — | 5·22* |
| 4 | — | — | — | 0·66* | 0·058 | — | — | — | 8·79* |
| 5 | 1·325 | — | 1·46 | 1·44* | 0·16 | 12·08 | — | 10·96 | 11·11* |
| 6 | 2·66 | — | — | 1·8 | 0·338 | 12·71 | — | — | 18·8 |
| 7 | 4·85 | — | — | — | 0·546 | 11·26 | — | — | — |
| 8 | 8·32 | 9·4 | 5·81 | 6·9 | 0·774 | 9·30 | 8·25 | 13·32 | 11·2 |
| 9 | 13·32 | 15·8 | 7·80 | — | 1·15 | 8·63 | 7·3 | 17·05 | — |
| 10 | 21·01 | — | — | 16·5 | 1·94 | 9·24 | — | — | 11·23 |
| 11 | 32·57 | 24·5 | 18·80 | — | 2·95 | 9·07 | 12·0 | 15·70 | — |
| 12 | 50·78 | 38·9 | — | 75·2 | 4·30 | 8·47 | 11·0 | — | 5·72 |
| 13 | 77·85 | — | 57·50 | — | 6·00 | 7·70 | — | 10·44 | — |
| 14 | 124·00 | 107·3 | 97·40 | 89·4 | 8·23 | 6·64 | 7·7 | 8·45 | 9·21 |
| 15 | 188·80 | — | 168·90 | — | 10·50 | 5·56 | — | 6·22 | — |
| 16 | 264·30 | 258 | 228·00 | 216·4 | 13·32 | 5·04 | 5·8 | 5·84 | 6·15 |
| 17 | 340·20 | 299 | 244·80 | — | 16·71 | 4·91 | 5·6 | 6·83 | — |
| 18 | 410·50 | — | 359·00 | 242·6 | 20·57 | 5·01 | — | 8·40 | 8·48 |
| 19 | 471·90 | 413 | 426·00 | — | 25·06 | 5·31 | 6·1 | 5·88 | — |
| 20 | 528·70 | 473 | 437·8 | 384·1 | 31·90 | 6·03 | 6·5 | 7·28 | 8·305 |

* These figures are those of Needham, the others in the column those of Sznerovna.

as have already been described in the Section on general metabolism. It would appear, therefore, that within the embryo there is a distinctly greater population of amino-acid molecules in the free state at some times than at others.

## 9·4. The Accumulation of Nitrogenous Waste-products

It is now time to turn to the engine room of the embryo, and to deal with the breakdown of the protein molecules which by their combustion furnish a supply of energy. The amount and intensity of combustion can be far more easily gauged in the case of protein than in the case of carbohydrate and fat, because of the incombustible residues left behind.

Urea may be taken first among the end products of protein combustion, although, as Fourcroy & Vauquelin found as long ago as 1805, by far the preponderant nitrogenous end product in the fowl is uric acid. Coindet was the first to find urea in avian urine, and in 1825 Prévost & Le Royer isolated from the allantoic fluid of a chick on the 17th day of development a substance which gave an insoluble compound with nitric acid, and which they identified with urea. In 1912 Fridericia carried out some experiments in which he estimated the quantity of urea in the allantoic fluid of the chick by the Schondorff method. Thus on the 17th day of development Fridericia obtained 4·5 mgm. urea per embryo, or 12 per cent. of the total excreted nitrogen (urea + uric acid). On the 20th day he got much less, only 1·7 mgm. urea per embryo, or 1·9 per cent. of the total excreted nitrogen. He concluded that only small and variable amounts of urea were present, and that the chick, like the adult hen, excreted all its nitrogen in the form of uric acid.

The subject merited re-examination however, and in 1925 I went into it anew. The method used was that of Folin & Wu, which, involving as it does the use of the specific enzyme urease, was more satisfactory than Schondorff's. The results obtained, applying the method to embryo + allantoic fluid + amniotic fluid, are shown graphically in Figs. 313 and 314. In Fig. 313 is seen the milligrams per cent. wet weight plotted against the time and also the milligrams per embryo. The latter rise steadily, as might be expected. The former rises steadily also, until the 9th day, at which point it becomes stationary for the rest of development. In other words, as far as the wet weight is concerned, the rate of production or excretion of urea

is very intense *after* the 4th day and *before* the 9th day. At later periods, although excretion of urea is still going on, it only just succeeds in keeping abreast of the wet weight. It was at once noteworthy that this intensive period of urea production occurred exactly between the carbohydrate period and the period associated with the predominance of fat metabolism.

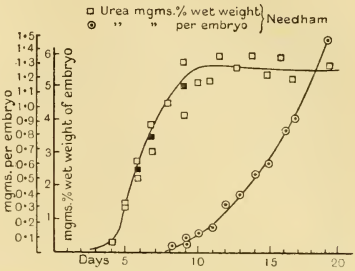



Fig. 313.

The effect may, of course, be due to other causes than to a specially intense combustion of protein during this period. For example, it may be due to a limiting factor such as the small size of the liver, or rather to the incapacity of the embryonic liver at this stage to turn urea into uric acid. That the developing liver can act in this way is probable from what we know of the desaturation process in embryonic metabolism (see p. 1171). If this were the case, however, an inflection in the curve of milligrams per cent. should appear at the time when the liver takes on this function. The activities of the enzyme arginase may also be involved (see p. 1312). But Kaieda has brought forward convincing evidence that the urea which the chick embryo produces is due to the deamination of protein breakdown-products. He injected each amino-acid into hen eggs before incubation and estimated the urea formed by the 16th day, with the following average results:

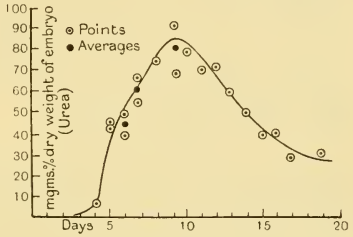



Fig. 314.

| | mgm. | |
|---|---|---|
| Control, Allantois | 1·00 | |
| ,, Embryo | 1·37 | |
| | 2·37 | |
| After injection, both | 3·60 | |
| Average increase | 1·23 | (ranging from 0·4 to 2·07 according to the amino-acid) |

The only amino-acids which failed to give an increase were cystein and serine: ammonium carbamate was effective but ammonium carbonate was not. Per mg. of injected substance the increase in urea was from 0·09 to 0·40 mgm.

In Fig. 314 the urea content is seen related to dry weight of embryo. Here we have a peak at the 9th day instead of an inflection; the urea excretion fails to keep pace with the increase in dry matter, and drops to a possibly constant level of 30 mgm. per cent. As before, it is the 9th day which is prominent.

Would the excretion of uric acid follow a similar course to a peak about half-way through development? Uric acid had been first reognised in the allantoic liquid of the chick embryo in 1819 when L. L. Jacobson of Copenhagen described it as at first clear and pale yellow, containing uric acid in solution, but later depositing urates in masses, which, he thought, also contained protein. "Diese Flüssigkeit", said Jacobson, "die in der ersten Tagen der Ausbrütung hell ist, wird nachher mehr zähe und schleimig, weisse Anhäufungen schwimmen in derselben; diese vermehren sich, worauf die wässerigten Teile verschwinden, so dass man in den letzten Tagen der Ausbrütung eine bedeutende Menge diese Anhäufungen in einem dicken und zähen Schleim gehällt, in der Allantois findet." Jacobson identified the uric acid by the murexide test. Jacobson's discovery was confirmed by Prévost & Le Royer in 1825, by Sacc in 1847 and by Stas in 1850.

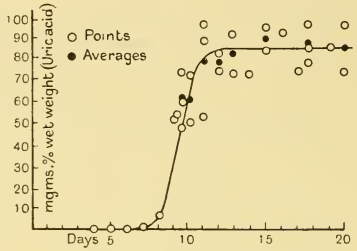


Fig. 315.

Two methods were used in estimating the uric acid in order to allow for the fact that, owing to the growth of the embryo, the whole scale of uric acid production is outside the best range of one method. As a micromethod for the early stages the colorimetric technique of Benedict & Franke was used, and for the later period the ammonium chloride precipitation method of Hopkins. The gradual increase in milligrams per embryo of uric acid made a very regular curve. In Fig. 315 is shown the milligrams per cent. wet weight, and here the significant plateau appears. In the first 7 days of develop-

ment the uric acid is exceedingly small in amount, but from the 7th to the 11th day it rises rapidly, until on the 12th day it attains a constant level which it does not leave. There is thus a specially intensive production of uric acid between the 7th and the 11th days of incubation.

Fig. 316 gives the uric acid in milligrams per cent. dry weight of embryo. The curve reaches a peak on the 11th day, after which it descends, and seems to be reaching a steady level by the time of hatching, at about 460 mgm. per cent. Its shape is the same as that found previously for urea. In Fig. 317 are shown the urea and the uric acid in milligrams per cent. wet weight of embryo plotted on the same scale. It shows the interesting fact that, on the 3rd, 4th, 5th, 6th and 7th days of incubation, the uric acid is rising distinctly more slowly than the urea, and, indeed, in absolute quantities per egg there is less uric acid than urea until the 8th day is reached. Between the 7th and the 8th day, the uric acid rises tremendously in amount, and, overtaking the urea, almost attains its final constant value. These relationships are better seen in Fig. 318, which gives the milligrams per cent. wet weight for both uric acid and urea, the abscissa being arranged so as to get them both on to the same graph. When this is done, it is obvious that, though both urea and uric acid rise in course of time to a constant level, the urea starts rising much earlier than the uric acid, and reaches its maximum level a day or so before. This is reflected on the milligrams per cent. dry weight curve, shown in Fig. 319, and it is seen that the urea is in advance of the uric acid by two days.

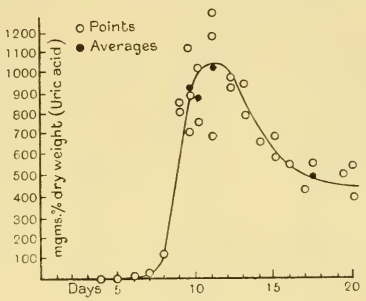


Fig. 316.

In the hen, as we have seen, the excreted nitrogen is mostly in the form of uric acid, and the urea takes only a very small share of it. At what stage in embryonic development is the adult relationship reached? In Table 137 and Fig. 320 the ratio uric acid/urea is seen. From the 14th day onwards it is constant at about 16, that being the adult level, but before the 7th day its value is less than unity, because more urea is present and more urea is daily excreted than uric

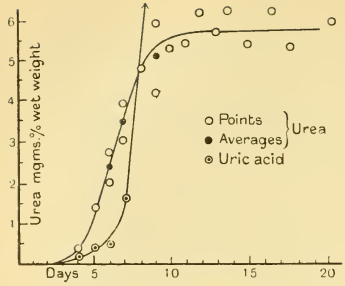



Fig. 317.

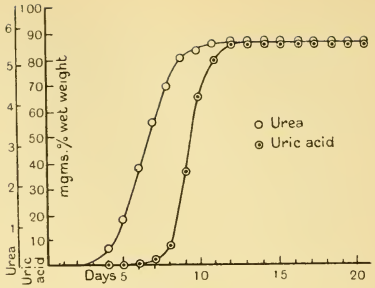



Fig. 318.

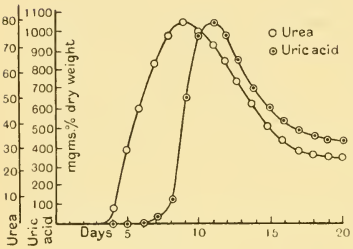



Fig. 319.

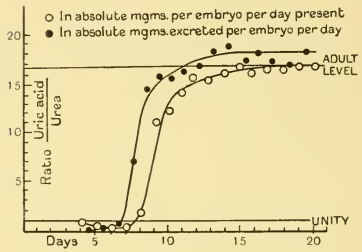



Fig. 320.

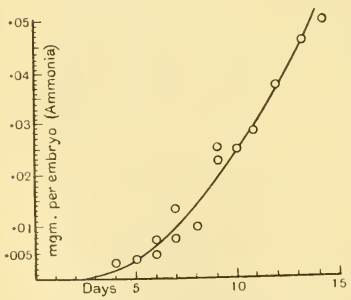



Fig. 321.

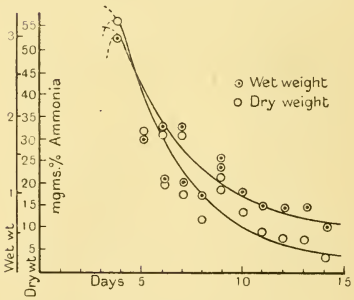



Fig. 322.

Figs. 318, 319 and 323 are smoothed curves.

acid. The adult ratio is therefore seen to be attained well before hatching.

Another nitrogenous excretory product remained to be estimated, namely, ammonia. It was improbable that this would have much effect on the picture of protein metabolism as a whole, but there was a possibility that it might have importance at some special time in embryonic life. The quantitative estimations were done by the Folin method, and led to the values shown graphically in Figs. 321 and 322.

Table 137. *Relations of uric acid and urea.*

| | Ratio: Uric acid/ urea milligrams per embryo | Ratio: Uric acid/ urea milligrams excreted per day per embryo | | Amounts excreted per day per embryo as % of the total nitrogen excreted per day per embryo* | |
|---|---|---|---|---|---|
| | | | | Uric acid nitrogen | Urea nitrogen |
| Day 4 | 0·8 | 0·145 | Day 4–5 | 9·4 | 90·6 |
| 5 | 0·2 | 0·210 | 5–6 | 13·21 | 86·79 |
| 6 | 0·2 | 0·604 | 6–7 | 26·39 | 73·61 |
| 7 | 0·45 | 7·18 | 7–8 | 83·43 | 16·57 |
| 8 | 1·63 | 13·52 | 8–9 | 90·70 | 9·30 |
| 9 | 10·80 | 14·89 | 9–10 | 91·44 | 8·56 |
| 10 | 11·80 | 14·92 | 10–11 | 91·44 | 8·56 |
| 11 | 13·10 | 15·92 | 11–12 | 91·90 | 8·10 |
| 12 | 14·31 | 16·28 | 12–13 | 92·10 | 7·90 |
| 13 | 14·29 | 17·28 | 13–14 | 92·52 | 7·48 |
| 14 | 15·15 | 17·96 | 14–15 | 92·76 | 7·24 |
| 15 | 15·73 | 16·88 | 15–16 | 92·34 | 7·66 |
| 16 | 16·03 | 17·03 | 16–17 | 92·48 | 7·52 |
| 17 | 15·70 | 16·69 | 17–18 | 92·28 | 7·72 |
| 18 | 15·77 | 16·55 | 18–19 | 93·22 | 6·78 |
| 19 | 16·00 | 17·65 | 19–20 | 92·68 | 7·32 |
| 20 | 16·50 | — | — | — | — |

* Assuming that no other nitrogen compounds are excreted.

For the increase in ammonia per embryo, the points lie on a well-defined curve, which is practically the same as regards its slope as those previously found for urea and uric acid (Figs. 313 and 328), but it will be seen that the total amounts with which we are now dealing are very much smaller than those of the urea curve, and infinitesimal compared with the amounts of uric acid which the embryo produces. Fig. 322 shows the amounts of ammonia present related to wet and dry weight of embryo. Inspection of this graph shows that the ammonia present in and around 100 gm. of embryo, falls steadily from the beginning of development. Whether the point at the 4th day represents a peak, or a descent from a yet higher value, we do not know. In Fig. 323 the amounts of ammonia,

urea and uric acid, expressed as milligrams per cent. of dry weight of embryo, have all been placed on the same graph. Here the comparison becomes very interesting, for, just as urea was previously found to rise to its maximum 2 days before uric acid, so now ammonia is at its maximum (or, more correctly, higher than at any other time so far determined) 5 days before urea. These time-relations between ammonia, urea and uric acid, during ontogenesis, are summarised in Table 138. The nitrogenous excretory product which has the smallest molecular weight and the highest nitrogen percentage reaches its maximum earliest in ontogenesis; that which has the largest molecular weight and the smallest nitrogen percentage reaches its maximum latest. The simplest product of deamination is the first to appear[1], the most complicated is the last. Yet the latter accounts for 91·5 per cent. of the total nitrogen excreted by an embryo throughout its development, and the former for only 1 per cent., while the intermediate compound, urea, accounts for 7·6 per cent.

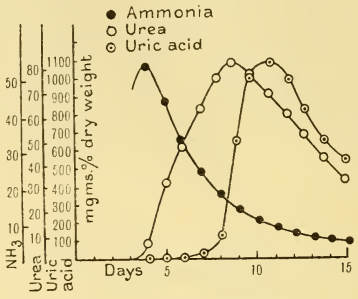



Fig. 323.

Table 138.

| | Molecular weight of compound | % of nitrogen in the compound | Time of peak of maximum production in the chick's development (in days) | Absolute milligrams nitrogen excreted during the whole development of the chick | % of the total nitrogen excreted during the whole development of the chick |
|---|---|---|---|---|---|
| Ammonia ... | 17 | 82·3 | 4 | 0·120 | 1·07 |
| Urea ... ... | 60 | 46·6 | 9 | 0·843 | 7·58 |
| Uric acid ... | 168 | 33·3 | 11 | 10·161 | 91·35 |
| | | Total ... ... ... | | 11·124 | |

A very interesting way of expressing the relationships here involved is shown in Fig. 324, where the milligrams of ammonia, urea, and uric acid nitrogen excreted per embryo per day are plotted against the time of development on semilog. paper. These curves correspond

[1] The ammonia may of course be derived from some substance other than protein, such as adenylic acid.

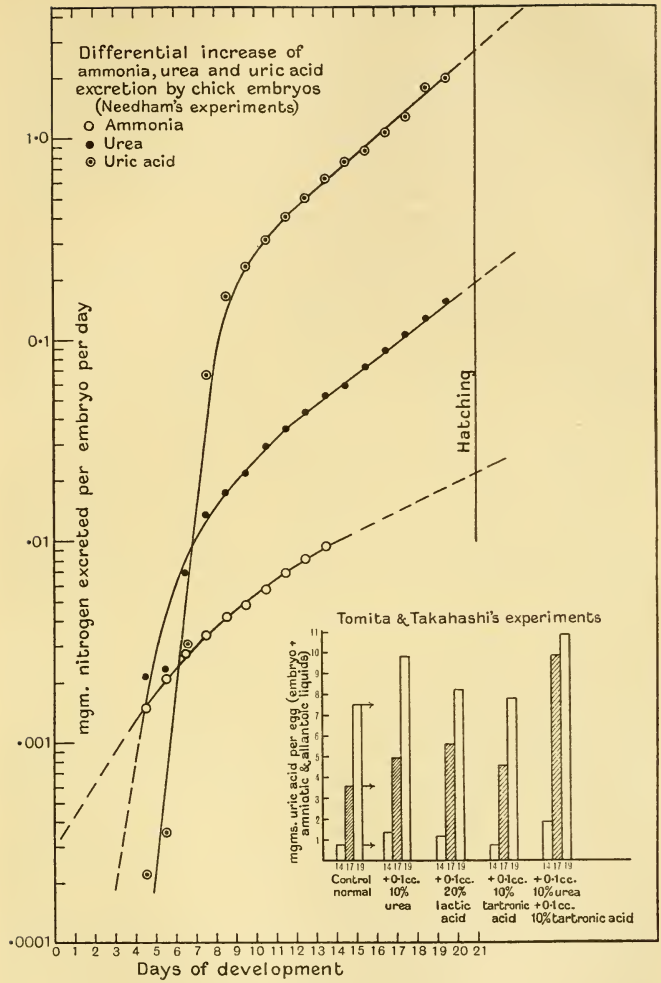

Differential increase of ammonia, urea and uric acid excretion by chick embryos (Needham's experiments)
Ammonia
Urea
Uric acid
mgm. nitrogen excreted per embryo per day
1·0
0·1
·01
·001
·0001
Hatching
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days of development
Tomita & Takahashi's experiments
mgms. uric acid per egg (embryo + amniotic & allantoic liquids)
14 17 19
Control normal
+0·1cc. 10% urea
+0·1cc. 20% lactic acid
+0·1cc. 10% tartronic acid
+0·1cc. 10% urea +0·1cc. 10% tartronic acid

Fig. 324.

to McDowell's log. weight/age graph described in Section 2·2 and illustrate well the differential growth-rates of the three functions. Ammonia nitrogen appears first but grows slowly and is soon overtaken by urea nitrogen, which in its turn is overhauled and passed by the uric acid nitrogen "growing" very rapidly, and finally reaching much larger proportions than any of the others. The graph also shows that finally the embryo has produced about ten times as much urea nitrogen as ammonia nitrogen and about ten times as much uric acid nitrogen as urea nitrogen.

There seems no reason to hesitate in classing this sequence among the most remarkable instances we possess of the occurrence of a recapitulation phenomenon in chemical embryology. To designate it thus does not, however, explain it and I shall return to the theory of recapitulation in the Epilegomena. The recent researches of Przylecki & Rogalski have thrown some light on the sequence of nitrogenous excretory compounds in the chick from another angle. In connection with Przylecki's wide investigations concerning the manner of excreting nitrogen which characterises different phyla and species, he directed his attention to the ontogenetic side, and enquired whether the development of the embryo involved the appearance or disappearance of such enzymes as uricase. For this purpose Przylecki & Rogalski used chick embryos to find out when the embryonic tissues can form uric acid, and when they can break it down. For the first series embryos were minced to an emulsion in water, glycerine and chloroform, and to some flasks xanthine was added, to others not. The results are shown in Table 139. Obviously if xanthine oxidase were present and active, the flasks to which the xanthine was added should show in all cases a higher uric acid content at the end of the experiment than those which had received no such addition. Here there is a turning-point between the 4th and 7th days of development, for before that time the xanthine flasks have no advantage over the others, but after that time an advantage is found without exception. This change-over point occurs at exactly the same time as the sudden rise of the control, which up to the 7th day reveals no preformed uric acid in the experimental material. The conclusion to be drawn then is that at some time between the 4th and the 7th day the embryonic xanthine oxidase awakens into activity, and the embryo acquires the power of forming uric acid from xanthine. If these time relations are compared with the appearance of uric acid shown in Fig. 317, the

correspondence is striking, but of course the greater part of the uric acid formed in normal life originates from ammonium lactate rather than from xanthine. Morgan's results agree with those of Przylecki & Rogalski, and were obtained by a different method (methylene blue). At the 7th day the yolk-sac and blastoderm gave a positive result, but yolk and white were negative. Xanthine oxidase was present in the kidney on the 15th day and rose extraordinarily sharply in the liver at the time of hatching.

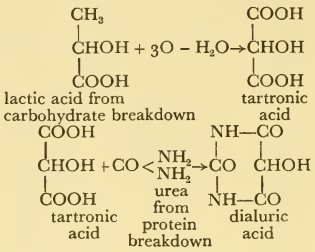
Table 139. *Przylecki & Rogalski's figures.*

| | Milligrams uric acid | | | |
|---|---|---|---|---|
| | | | Flasks to which 15 mgm. uric acid was added | |
| Age in days | Flasks alone | Flasks to which 25 mgm. xanthine was added | Heated at 100° | Not heated |
| 2 | 0·0 | 0·0 | 6·5 | 4·1 |
| | 0·0 | 0·0 | 7·0 | 5·5 |
| | 0·0 | 0·0 | 2·8 | 1·8 |
| 4 | 0·0 | 0·0 | 6·7 | 6·0 |
| | 0·0 | 0·0 | 6·0 | 5·2 |
| 7 | 1·5 | 1·8 | 6·0 | 5·1 |
| | 1·4 | 1·9 | 5·3 | 4·4 |
| | 2·0 | 2·5 | 5·5 | 5·1 |
| 10 | 3·0 | 3·5 | 4·5 | 4·5 |
| | 2·8 | 3·6 | 5·1 | 5·1 |
| | 2·7 | 3·7 | 5·5 | 5·5 |
| 14 | 2·5 | 3·4 | 7·0 | 7·0 |
| | 3·5 | 4·4 | 4·1 | 4·1 |
| | 3·1 | 4·6 | 3·9 | 3·9 |
| 21 | 2·4 | 3·7 | 8·0 | 8·0 |
| | 1·8 | 3·4 | 4·3 | 4·3 |
| | 2·6 | 3·6 | 4·0 | 4·0 |

Przylecki & Rogalski not only found that before the time at which uric acid appears in the excreta the embryo has no power of forming uric acid, but also that it destroys it if provided. The right-hand part of Table 139 makes this clear, for in the early stages of development a heated emulsion of embryonic tissues with added uric acid always contains more of this substance at the end of the experiment than a parallel flask which has not been heated. Between the 7th and 10th days, however, a change takes place, and, after that time, the presence of uricase can no longer be demonstrated. It is plain that the facts revealed by the Polish workers fit in well with those described above. But the fact that the uricase does not disappear until the 10th day may mean that before that time uric acid is formed from substances such as ammonium lactate and equally rapidly broken down to allantoin.

Allantoin should therefore be estimated quantitatively in the allantois during the first week of incubation. Przylecki & Rogalski's findings with regard to the appearance of xanthine oxidase are in accord with those of the American school described in Section 14·10.

The only information as to the way in which the avian uric acid is formed in the egg is due to the researches of Tomita & Takahashi. Believing that the following chain of reactions took place:




dialuric acid + urea → uric acid,

they injected tartronic acid and urea into hen's eggs and succeeded in obtaining an increased formation of uric acid by the embryo, as shown in Fig. 324. But some doubt may be expressed concerning their control of the range of variation in normal eggs and Clementi considers that urea cannot be an intermediate step in the synthesis of sauropsid uric acid.

## 9·5. Protein Catabolism

We must now return to the question of protein combustion in the chick. Knowing the amounts of ammonia, urea and uric acid present each day during development, it is simple to calculate the amount of protein burned. This gives the graph shown in Fig. 325, in which the milligrams of protein combusted by 100 gm. of dry weight of embryo per day are plotted against the time in days. A marked peak appears at 8·5 days' development, the significance of which is sufficiently indicated by the labels summarising other evidence. This has been discussed in Section 7·7. One may say that a given weight of embryo combusts 6 or 7 times as much protein on the 8th day of development as it does on the 4th or the 16th day. The combustion of protein thus goes on during both the carbohydrate and fat periods,

but not to anything like the same extent as it does when the former is passing over into the latter.

Rapkine has pointed out that this peak in protein catabolism precisely coincides with the time of development at which chick embryos are most suitable for use in providing the plasma for tissue cultures. Whether the growth-producing substance is a proteose, a peptone, a dipeptide, or even an amino-acid, it does at any rate seem clear that it is a nitrogenous substance of protein origin, and it is therefore interesting to note that it seems to be at its greatest concentration in the embryo just at the time when there is a maximum of protein catabolism. Carrel gives the 7th to the 10th day as the period most suitable for taking embryos for this purpose, and the peak, as we have seen, occurs at 8·5 days of development. It is also very interesting that Remotti's curve for the activity of the yolk proteases reaches its maximum on the 9th day of development (see Fig. 423 *a*). This brings up the whole question of how the yolk and white proteins are transformed into the tissue proteins during development. It is possible that the former are not reduced to their constituent amino-acids entirely, but only to a proteose or peptone stage. If this were so, Carrel's growth-promoting substance might simply be one of the normal intermediate products. There have not so far been any researches designed to test this interesting possibility.

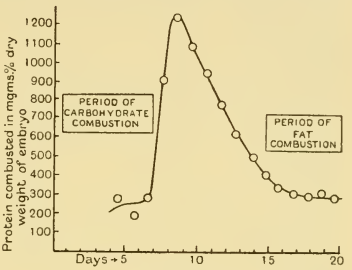



Fig. 325.

From the data in Table 138, it will be seen that the total number of milligrams of nitrogen excreted by the embryo during its development (or, more accurately, transmuted into the nitrogenous waste products, urea and uric acid) is 11·00. Sendju, as we have seen, estimated the total amounts of various amino-acids in the hen's egg during the course of its development, finding a very slight increase in the histidine, and a constancy in the arginine, lysine, and mono-amino-acids, while in the tryptophane and tryosine, on the other hand, there was a considerable falling off. He correlated this with the formation of pigments, and did not take into account losses by

combustion for the production of energy. According to Sendju, the tryptophane per egg descends from 134 mgm. to 60, a loss of 74 mgm.; and the tyrosine, beginning at 420 mgm., falls to 260, this being a loss of 160 mgm.

| | Nitrogen (mgm.) |
|---|---|
| 74 mgm. tryptophane contain ... | 9·868 |
| 160 mgm. tyrosine contain ... ... | 12·374 |
| Total ... ... | 22·242 |

Supposing, then, that these two amino-acids are the only ones, or even the principal ones combusted during development, it would follow that about 50 per cent. was burnt and 50 per cent. was available for the work of haemoglobin formation or other uses. It is certainly strange that tyrosine and tryptophane should be precursors of a purine, but it must be remembered that in the bird uric acid corresponds to the urea of mammals, and is produced from the ammonia due to deamination of combusted proteins.

It is interesting to compare the estimates of protein catabolism obtained by assessing the coagulable protein disappearing with those obtained by assessing the total nitrogen excreted. Sakuragi found that the coagulable protein diminished during development from 1846 mgm. of nitrogen per cent. to 1698 (see Table 135). 148 mgm. are therefore lost per cent. of the contents of an average egg, which amounts to 67 mgm. per individual egg. Yet none of the investigators who have estimated the excreted nitrogen have recovered a corresponding amount:

| | | Milligrams nitrogen per embryo |
|---|---|---|
| Coagulable nitrogen disappearing ... | Sakuragi | 67 |
| Waste products appearing (in nitrogen) | Fiske & Boyden | 36 |
| | Fridericia | 23 |
| | Needham | 12 |
| | Targonski | 8 |
| | Kamei | 1 |

There is thus some reason for believing, whichever of these sets of data turns out to be nearest the truth, that the protein combusted is probably not more than a third of the coagulable protein disappearing. The fate of the rest still remains doubtful. Whether the ovomucoid takes part in combustion is a question at present unanswerable; all that can be said is that there is no preferential absorption of ovomucoid as against ovoalbumen from the white.

We can now examine again the part played by ammonia, urea and uric acid as excretory products. Another way of expressing the relationship leads to the result shown in Fig. 326. Here, assuming that the ammonia, urea and uric acid together make up the total excreted nitrogen (which is not quite true), the partition between them has been calculated as milligrams excreted by the embryo each day in per cent. of the total nitrogen excreted by the embryo each day. Between the 4th and 5th days, the uric acid only accounts for 9 per cent. of the total nitrogen, but so rapidly does the change occur that, between the 8th and 9th days, it accounts for as

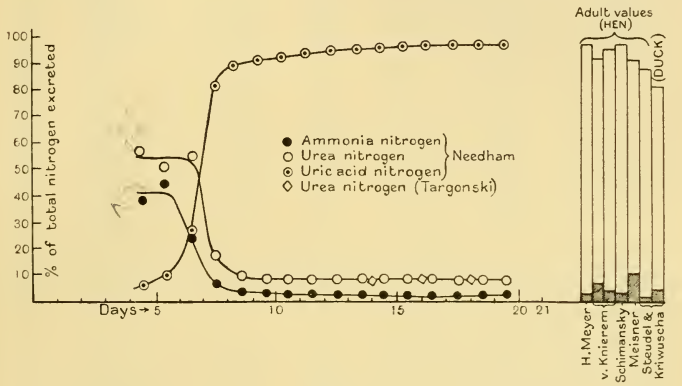



Fig. 326.

much as 90·7 per cent. It has therefore practically reached its adult level, as is shown by the comparative standards to the right of the graph. The shaded parts at the bottom represent urea and the rest uric acid; they are figures taken from Meyer; von Knierem; Schimansky; Meissner, and Steudel & Kriwuscha.

It is clear that in the first week of development the relationship of urea and ammonia to uric acid is altogether different from what holds in the adult, but that in the last two weeks of development the adult value is rigidly adhered to. These results throw light on the fact that the allantoic fluid in the chick passes from *p*H 7·2 to *p*H 6·0 in the last half of incubation, whereas, before the 9th day, it has been constant at about *p*H 7·2 (see Fig. 211).

In 1921 Sznerovna made estimations of the nitrogen contained in the body of the embryo, and that contained in the allantoic fluid at different stages of incubation. She found that the ratio of these,

Table 140.

| | Milligrams nitrogen present in embryo each day (cumulative) | | | Milligrams nitrogen excreted by the embryo up to each day (cumulative) | | Sznerovna's ratio (Nitrogen in embryo/Nitrogen excreted) | | |
|---|---|---|---|---|---|---|---|---|
| | Sznerovna | Murray | LeBreton & Schaeffer | Sznerovna | Needham | Sznerovna | Murray | LeBreton |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Day 5 | — | 1·325 | 1·46 | — | 0·00234 | — | 566·2 | 624·0 |
| 6 | — | 2·661 | 2·1 | — | 0·00500 | — | 533·4 | 420·9 |
| 7 | — | 4·850 | 4·0 | — | 0·0149 | — | 325·6 | 260·6 |
| 8 | — | 8·323 | 6·5 | — | 0·095 | — | 106·5 | 68·3 |
| 9 | — | 13·32 | 8·0 | — | 0·279 | — | 47·7 | 28·7 |
| 10 | 16·5 | 21·01 | 12·5 | 0·95 | 0·5344 | 17·5 | 39·32 | 23·4 |
| 11 | — | 32·57 | 18·8 | — | 0·8772 | — | 37·13 | 21·4 |
| 12 | 75·2 | 50·78 | 32·0 | 4·2 | 1·3161 | 18·1 | 38·59 | 24·3 |
| 13 | — | 77·85 | 57·5 | — | 1·8699 | — | 41·64 | 30·8 |
| 14 | 89·4 | 124·00 | 102·0 | 5·5 | 2·5546 | 16·4 | 48·53 | 39·9 |
| 15 | — | 188·80 | 154·0 | — | 3·3810 | — | 55·86 | 45·5 |
| 16 | 216·4 | 264·30 | 212·0 | 14·1 | 4·3376 | 15·3 | 60·94 | 48·9 |
| 17 | — | 340·20 | 265·0 | — | 5·5101 | — | 61·74 | 48·1 |
| 18 | 242·6 | 410·50 | 322·0 | 20·1 | 6·9155 | 20·1 | 59·35 | 46·6 |
| 19 | — | 471·90 | 390·0 | — | 8·8452 | — | 53·55 | 44·1 |
| 20 | 384·1 | 528·70 | 475·0 | 23·8 | 11·0038 | 16·1 | 48·07 | 34·3 |

i.e. nitrogen in embryo/nitrogen in allantoic fluid, was practically a constant, wavering round about 17 (see Table 140). In other words, for every 1 gm. of nitrogen in the allantoic fluid, there were to be found 17 grams of nitrogen in the body of the embryo. Her estimations did not begin before the 10th day, so in view of the relations which we have already found to hold between the early and late periods of development, a recalculation of her ratio was desirable.

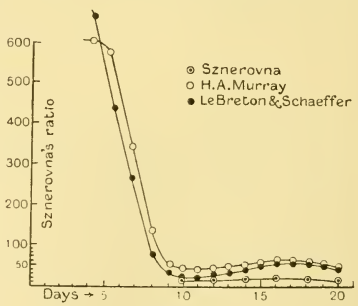


Fig. 327.

It was assumed that only small errors would be introduced by calling the urea nitrogen plus the uric acid nitrogen the nitrogen present in the allantoic fluid, and two different sets of figures for the total nitrogen in the embryo were available. The important difference between the nitrogen figures

of LeBreton & Schaeffer, on the one hand, and Murray, on the other, is that the former excluded the membranes in their estimations while the latter probably included them. We therefore have a way

Table 141.

Uric acid milligrams per embryo

| | Needham | Fridericia | Fiske & Boyden | Kamei | Targonski | Tomita & Takahashi |
|---|---|---|---|---|---|---|
| Day | Embryo + amnios + allantois (Benedict & Franke's colorimetric method; Hopkins' ammonium chloride method) | Amnios + allantois (Salkovski's silver precipitation method) | Allantois only (Folin's colorimetric method) | Allantois only (Folin & Wu's colorimetric method) | Allantois only (Morris & Macleod's colorimetric method) | Embryo + amnios + allantois (His & Hagen's extraction and Folin's colorimetric method) |
| 0 | — | — | — | — | — | — |
| 1 | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — |
| 3 | — | — | 0·007 | — | — | — |
| 4 | 0·00029 | — | 0·030 | — | — | — |
| 5 | 0·00095 | — | 0·055 | — | — | — |
| 6 | 0·0020 | — | 0·33 | — | — | — |
| 7 | 0·0116 | — | 0·72 | — | — | — |
| | 0·0116 | — | — | — | — | — |
| 8 | 0·0845 | — | 1·77 | — | 0·503 | — |
| | 0·0845 | — | — | — | — | — |
| 9 | 0·950 | — | 2·87 | 0·266 | — | — |
| | 0·969 | — | — | — | — | — |
| 9–10 | 1·301 | — | — | — | — | — |
| 10 | 1·873 | — | 4·33 | — | 2·06 | — |
| | 1·344 | — | — | — | — | — |
| 11 | 3·410 | 3·5 | 7·1 | — | — | — |
| | 3·733 | — | — | — | — | — |
| | 1·917 | — | — | — | — | — |
| 12 | 4·276 | 5·2 | 10·61 | — | 3·51 | — |
| | 4·077 | — | — | — | — | — |
| 13 | 5·314 | 5·5 | 15·06 | — | — | — |
| | 6·330 | — | — | — | — | — |
| 14 | 6·930 | 11·7 | — | 0·507 | 4·75 | 0·55 |
| 15 | 10·760 | 18·6 | — | — | — | — |
| | 9·920 | — | — | — | — | — |
| 16 | 13·650 | 28·8 | — | — | 9·30 | — |
| 17 | 13·940 | 42·8 | — | 3·11 | — | 3·50 |
| 17–18 | 17·640 | — | — | — | 13·74 | — |
| 18 | — | 49·7 | — | — | — | — |
| 19 | — | — | — | — | — | 7·50 |
| 19·5 | 26·090 | 57·4 | 100·0 | — | — | — |
| 20 | 32·700 | — | — | — | — | — |
| | 25·600 | 64·8 | — | — | — | — |

of determining what part the membranes are playing in the protein metabolism. These relations are shown in the form of a graph in Fig. 327. The newly calculated ratio does not quite become a constant during the last 10 days of incubation, although it approximates

to one, and it never reaches the low figure obtained by Sznerovna. The extreme smallness of the protein catabolism during the first 6 or 7 days is reflected on this curve in the extreme height of the ratio,

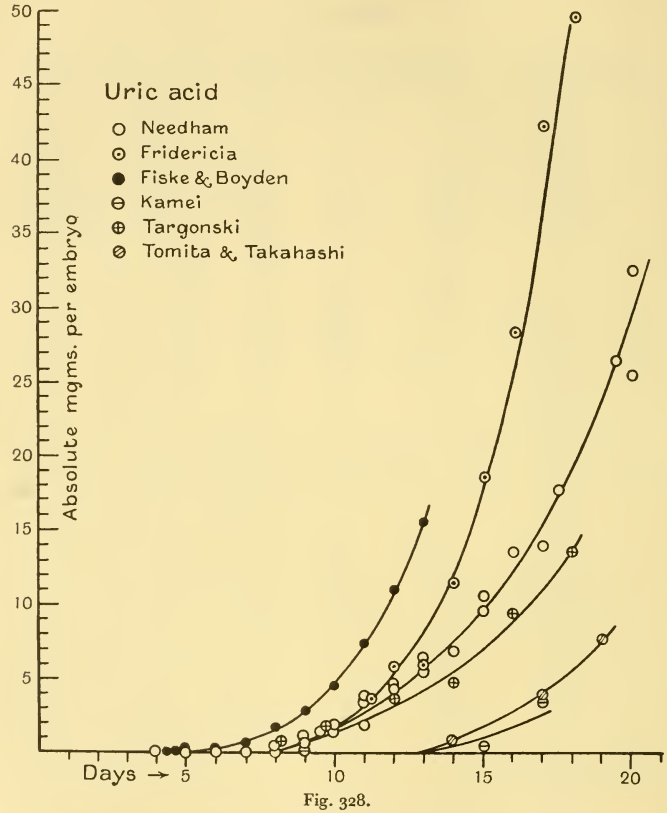



Fig. 328.

but Sznerovna missed the early descent. The metabolism of protein seems to be more intense in the absence of the membranes, but it is perhaps legitimate to conclude that the part they play in the combustion of proteins is slight.

Among the investigators who have estimated the uric acid produced by the chick embryo there is some divergence in the absolute magnitude of their values. Table 141 illustrates this, and shows that Fridericia's results, for instance, though at first of exactly the same order as mine, draw off about the 15th day, and rise much higher during the remaining time. Fiske & Boyden's, on the contrary, are always higher than either Fridericia's or mine, while Kamei's are the lowest of all. Targonski embodied his results in singularly confusing and obscure tables, but the uric acid production of his embryos can be calculated from them, with the result that his data confirm mine, none of his points being very far away from the curve of Fig. 328. These are all the sets of figures for uric acid production which we have at present, and, until a thorough comparative assessment is made of the effect of breed, etc., on uric acid production, the differences will remain difficult to account for[1]. But it is important that the peak in nitrogen catabolism emerges from at least two of them, and it may be supposed that this is due to a relative rather than a universal validity inhering in each series. This is illustrated by Fig. 329.

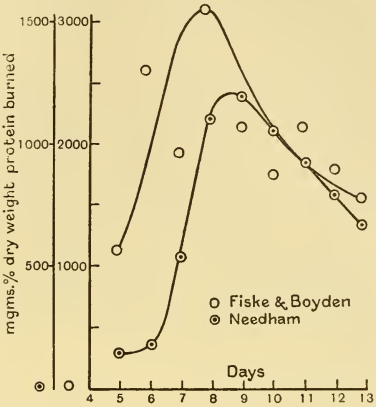



Fig. 329.

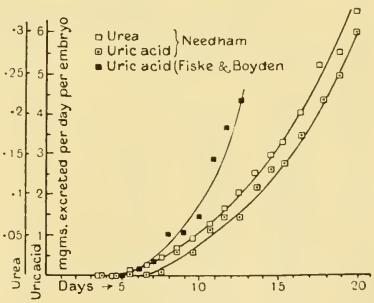



Fig. 330.

[1] Differences of technique may account for much divergence; thus some methods estimate ergothioneine as well as uric acid. Calvery's later figures for uric acid agree exactly with those of Needham.

The data of Fiske & Boyden and of Needham, when plotted in terms of milligrams of protein combusted each day by 100 gm. dry weight of embryo, show the peak in both cases. Fridericia's points, according to Cahn, confirm the downward trend from the 11th day onwards.

It will be noticed from Table 141 that in my set of figures the uric acid in the embryonic body is included in the estimations. I did no experiments to determine the degree of uric acid retention, but Fiske & Boyden reported that, between the 5th and 11th days, the uric acid content of the embryonic body never exceeds 2 mgm. per cent. Between the 7th and 8th days, then, when the embryo weighs about a gram (wet weight), 0·02 mgm. uric acid would be contained in it, although during this time 0·5 mgm. would be excreted.

The data for the daily excretion of uric acid—a necessary step in these calculations—are shown in Fig. 330. Fiske & Boyden attributed significance to the irregularities in the points of their curve, but for this there is no warrant.

Table 142. *Allantoic liquid* (*milligrams per egg*).

| | Fiske & Boyden | | | | | Targonski | | |
|---|---|---|---|---|---|---|---|---|
| Day | Total nitrogen | Uric acid nitrogen | Nitrogen other than uric acid (residual nitrogen) | Sznerovna. Total nitrogen | Kamei. Total nitrogen | Total nitrogen | Uric acid nitrogen | Nitrogen other than uric acid (residual nitrogen) |
| 1 | — | — | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — | — | — |
| 3 | — | — | — | — | — | — | — | — |
| 4 | — | — | — | — | — | — | — | — |
| 4·5 | 0·034 | 0·002 | 0·032 | — | — | — | — | — |
| 4·8 | 0·068 | 0·01 | 0·058 | — | — | — | — | — |
| 5 | 0·10 | 0·02 | 0·08 | — | — | — | — | — |
| 6 | 0·92 | 0·11 | 0·18 | — | — | — | — | — |
| 7 | 0·45 | 0·24 | 0·21 | — | — | — | — | — |
| 8 | 0·89 | 0·59 | 0·30 | — | — | 0·71 | 0·17 | 0·54 |
| 9 | 1·52 | 0·96 | 0·56 | — | 4·81 | — | — | — |
| 10 | 2·29 | 1·44 | 0·85 | 0·95 | — | 1·84 | 0·67 | 1·17 |
| 11 | 3·38 | 2·37 | 1·01 | — | — | — | — | — |
| 12 | 4·81 | 2·54 | 1·27 | 2·8 | — | 2·77 | 1·08 | 1·69 |
| 13 | 7·35 | 5·02 | 2·33 | — | — | — | — | — |
| 14 | — | — | — | 5·5 | 52·74 | 3·19 | 1·82 | 2·37 |
| 15 | — | — | — | — | — | — | — | — |
| 16 | — | — | — | 14·1 | — | 3·75 | 3·65 | 0·10 |
| 17 | — | — | — | — | 13·44 | — | — | — |
| 18 | — | — | — | 17·9 | — | 9·65 | 5·65 | 4·00 |
| 19 | — | — | — | — | — | — | — | — |
| 20 | — | — | — | — | — | — | — | — |

## 9·6. Nitrogen-excretion; Mesonephros, Allantois and Amnios

The total nitrogen in the allantoic fluid has been measured quantitatively by Fiske & Boyden; Sznerovna; Kamei, and Targonski. Kamei's figures are obviously aberrant, but the rest correspond more or less closely, as an inspection of Table 142 indicates well enough. The principal interest of it is the fact, emerging from Fiske & Boyden's work, that at the earlier stages of development uric acid is by no means the predominating constituent of the nitrogen excretion. The uric acid rises above the rest, however, at the 7th day of development. This confirmed very strikingly my original finding, and the harmony of the results is shown in the comparison of Figs. 326 and 331. Targonski's more erratic figures also show the typical cross-over in the importance of uric acid and other nitrogenous molecules as excretory products.

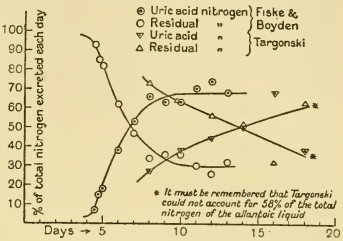



Fig. 331.

What *is* the residual nitrogen of the early stages? A balance sheet can be drawn up as follows:

Table 143. *Allantoic liquid* (*milligrams per embryo*).

| | Fiske & Boyden | | | | Needham | | | |
|---|---|---|---|---|---|---|---|---|
| Day | Total nitrogen | Uric acid nitrogen | Creatine nitrogen | Amino-acid nitrogen | Urea nitrogen | Ammonia nitrogen | Unaccounted for | % unaccounted for |
| 4·0 | — | — | — | — | 0·0002 | 0·0033 | — | — |
| 4·8 | 0·082 | 0·011 | 0·001 | — | — | — | — | — |
| 5·0 | 0·107 | 0·017 | 0·007 | 0·047 | 0·0025 | 0·004 | 0·03 | 28 |
| 5·1 | 0·120 | 0·025 | 0·008 | 0·058 | — | — | 0·02 | 17 |
| 6·0 | — | — | — | — | 0·005 | 0·006 | — | — |
| 8·0 | — | — | — | — | 0·026 | 0·014 | 0·33 | 26 |
| 8·2 | 1·27 | 0·86 | 0·039 | — | — | — | | |
| 9·9 | 2·32 | 1·82 | — | 0·28 | — | — | 0·13 | 6 |
| 10·0 | — | — | — | — | 0·068 | 0·025 | | |
| 12·9 | 6·74 | 5·21 | — | 0·47 | — | — | 0·82 | 12 |
| 13·0 | — | — | — | — | 0·192 | 0·048 | | |

From this it is clear that an amount of nitrogen varying from 6 to 28 per cent. of that contained in the allantoic liquid is as yet unaccounted for. Examination of the table shows the constant presence

of creatine and amino-acid nitrogen, which latter in the early stages accounts for as much as 40 per cent. of the total nitrogen[1].

Although the absolute amounts of the excreted nitrogenous waste products are the values which it is most important to know, the concentration of them in the allantoic liquid is of some interest. Here the most complete series of estimations is that of Fiske & Boyden, plotted in Fig. 332. The vertical lines show the range of variation in their figures, and the white and black circles refer to Targonski's points. At the beginning the composition of the allantoic fluid, as regards its nitrogenous crystalloids, quite closely resembles the concentrations found in the plasma of adults. Fiske & Boyden regard it as a mere filtrate till the end of the 5th day. After that time the concentration of uric acid steadily rises, at least until the end of the 13th day. The concentration of the residual nitrogen, on the contrary, falls markedly until the 9th or 10th day, as would be the case if the formation of any quantity of urea and ammonia was suspended when the formation of uric acid began, and afterwards rises again, as would be the case if other substances, such as amino-acids or creatinine, began to enter into it. About the end of the 2nd week of incubation the allantoic fluid acquires more urates than it can dissolve—thus it is sometimes milky as early as the 7th day, and sometimes clear as late as the 13th. Besides this turbidity, the urates are deposited as slimy stringy masses, which, though at first soft, eventually become hard and almost brittle. Fiske & Boyden observed that as much as 87 per cent. of the total uric acid present may be contained in these deposits. Boyden has drawn attention to the blister-shaped vesicles which often, if not always, occur within the inner wall of the chick's allantois in the region of the amnio-

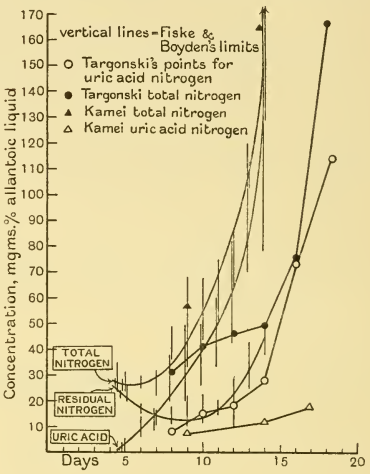



Fig. 332.

[1] See the foot-note on p. 977 and Table 163.

allantoic fusion. These first appear about the time that the lymphatic circulation is established in the allantois (7th day), and increase in number and size till the end of development. Gentle heating coagulates the liquid inside the blisters. They may either be concerned with the absorption of protein, or they may be caused by the sharp edges of the uric acid crystals, or, again, they may secrete mucin. As regards the excretion of urates, Fiske & Boyden pointed out that the anatomical "evidence" that the mesonephros is functionally inert is no more than an expression of opinion, and that all we know about the presence of nitrogenous waste products in the allantois goes to show that it is functional. That water passes through the mesonephros even against pressure as early as 2·5 days of incubation was proved by the experiments of Boyden, who obstructed the Wolffian duct, and afterwards observed hydronephrosis. Indeed, without the mechanical distention produced by the excretion of the mesonephros the allantois fails to grow normally in size, and consequently the proper respiratory apparatus of the allantoic vessels does not develop. And the mesonephros is alone in a position to dispose of waste products up till the 11th day. Aggazzotti, in his work on the *p*H of the allantoic liquid (see Fig. 211), concluded that the allantoic liquid was not a true excretion before the 11th day, because it was not acid, although the yolk was, but, as Fiske & Boyden point out, no one could have predicted *a priori* what the reaction of the embryonic excreta would be.

Fridericia was much interested in the problem of relating morphological knowledge about such organs as the liver and the mesonephros to the results obtained on uric acid excretion. For the former organ he could find no hint in the literature, but he made an attack on the mesonephros by measuring its dimensions in a large number of embryos. In the chick embryo the mesonephros is a large yellowish-red organ on the 16th day, but as it hands over its functions to the metanephros at a later stage it atrophies, and by the 20th day is small, thin and pale. Fridericia's measurements of its size are shown in Fig. 333, from which it appears that the mesonephros reaches its greatest size upon the 16th day. Fridericia related this to the peak which his figures for daily uric acid excretion show at that point of development, but, as the only other set of data covering it do not show such a falling-off (Needham), it is doubtful if any emphasis can be laid upon it.

A good deal of light has been thrown on the functioning of the embryonic excretory organs in the chick by the injection of vital dyes into the egg during development. The pioneer in this line of work was Bakounine, who in 1895 injected indigo-carmine intravenously into chick embryos varying from 3 to 15 days' development, and in all cases observed the dye in the cells of the proximal portion of the mesonephric tubules. Undoubtedly excretion of the stain was occurring. Zaretzki was the first to inject dyes into the air-space. He used trypan blue, trypan red, neutral red, methylene blue, fluorescein, eosin and ethyl green. With trypan blue there was in the late stages of incubation a slight colouring of the amniotic liquid, but not of the embryo. When the dye was injected into the outer wall of the allantois, he observed a diffuse staining of the embryo, amniotic liquid and foetal membranes. These results were rather difficult to interpret, as were those later obtained by Gräper, who unsuccessfully tried to use an *in vitro* technique for keeping stained whole blastoderms alive outside the egg. Hanan has shown more recently that trypan blue, once within the circulation of the embryo, is excreted through the kidneys, and appears in the allantoic but not in the amniotic liquid, strongly suggesting that, in the case of birds, foetal urinary water does not contribute to the formation of the latter. The excretory activity of the mesonephros, he found, began on the 4th day of incubation, which agrees very well with the earlier results of Bakounine and of Wislocki, and with the fact established by Boyden that hydronephrosis cannot easily be produced before the 4th day. The more recent researches of Atwell & Hanan and of Hurd with trypan blue have established (if the behaviour of the dye in the cells is any criterion) that the excretory activity of the mesonephros begins on the 4th day, and goes on alone till the 11th day, at which time the metanephros comes into operation. Both function together till the

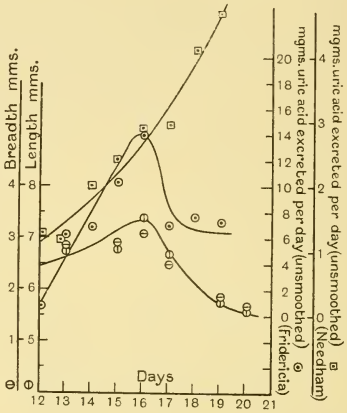



Fig. 333.

18th day, after which the metanephros continues by itself. For experiments on the excretory powers of the mesonephros in the salamander, *Necturus maculosus*, see the papers of Dawson and of White & Schmitt.

Table 144. *Concentration of constituents in the allantoic liquid of the chick.* (*Milligrams* $^{0}/_{0}$.)

| | | Kamei | | | | | Targonski | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | Fiske & Boyden Total nitrogen | Total non-protein nitrogen | Non-protein nitrogen (non-basic) | Non-protein nitrogen (basic) | Ammonia | Urea | Total nitrogen | Non-protein nitrogen (basic) | Non-protein nitrogen (non-basic) | % of total non-protein nitrogen (basic) | % of total non-protein nitrogen (non-basic) |
| 0 | — | — | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | — | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — | — | — | — | — | — |
| 3 | — | — | — | — | — | — | — | — | — | — | — |
| 4 | 28 | — | — | — | — | — | — | — | — | — | — |
| 5 | 26 | — | — | — | — | — | — | — | — | — | — |
| 6 | 27 | — | — | — | — | — | — | — | — | — | — |
| 7 | 29 | — | — | — | — | — | — | — | — | — | — |
| 8 | 34 | — | — | — | — | — | — | — | — | — | — |
| 9 | 43 | 4·6 | 3·6 | 1·1 | 0·8 | 5·6 | — | — | — | — | — |
| 10 | 55 | — | — | — | — | — | — | — | — | — | — |
| 11 | 68 | — | — | — | — | — | 46 | 18 | — | 40·2 | 59·8 |
| 12 | 85 | — | — | — | — | — | — | — | — | — | — |
| 13 | 106 | — | — | — | — | — | — | — | — | — | — |
| 14 | 151 | 41·6 | 0·6 | 4·7 | 3·6 | 21·2 | 85 | 43 | 42 | 51·0 | 49·0 |
| 15 | — | — | — | — | — | — | — | — | — | — | — |
| 16 | — | — | — | — | — | — | 92 | 60 | 32 | 65·1 | 34·9 |
| 17 | — | 87·1 | 20·7 | 8·5 | 12·5 | 56·7 | — | — | — | — | — |
| 18 | — | — | — | — | — | — | 116 | 50 | — | 43·2 | 56·8 |
| 19 | — | — | — | — | — | — | — | — | — | — | — |
| 20 | — | — | — | — | — | — | — | — | — | — | — |

In the previous discussion, nothing has been said about the other estimates of the urea present in the allantoic fluid. Kamei made a few determinations of ammonia and urea, and the figures are shown in Table 144. On the 14th day he found 21·2 mgm. per cent. of urea in the allantoic fluid, or, taking its volume at 6·5 c.c., 1·28 mgm. absolute, and on the same day 3·4 mgm. per cent. urea in the amniotic fluid, or, its volume being roughly 2 c.c., 0·068 mgm. absolute. The total amount present per embryo was therefore 1·35 mgm., a value somewhat in excess of my 14th day determination, namely, 0·5 mgm. Kamei did not state what method he used for his urea determinations. In the same way his estimate of 0·276 mgm. of ammonia in the amniotic and allantoic liquids on the 14th day

exceeds my value of 0·06 mgm. for amniotic and allantoic liquids and embryo. Such discrepancies seem to be inevitable in the first approximations, and can only be abolished by further work. Kamei's figures for the non-protein nitrogen fractions are not easy to interpret.

Table 145. *Concentration of constituents in the amniotic liquid of the chick.* (*Milligrams* %.)

| | Fiske & Boyden | | Kamei | | | | | | Targonski | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | Total nitrogen | Uric acid nitrogen | Total nitrogen | Total non-protein nitrogen | Non-protein nitrogen (non-basic) | Non-protein nitrogen (basic) | Ammonia | Urea | Total nitrogen | Protein nitrogen | Non-protein nitrogen |
| 0 | — | — | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | — | — | — | — | — | — | — |
| 2 | — | — | — | — | — | — | — | — | — | — | — |
| 3 | — | — | — | — | — | — | — | — | — | — | — |
| 4 | — | — | — | — | — | — | — | — | — | — | — |
| 5 | — | — | — | — | — | — | — | — | — | — | — |
| 6 | — | 0·10 | — | — | — | — | — | — | — | — | — |
| 7 | 2·9 | — | — | — | — | — | — | — | — | — | — |
| 8 | — | — | — | — | — | — | — | — | — | — | — |
| 9 | — | 0·10 | 11·9 | 33·1 | 1·5 | 0·6 | 0·8 | 3·4 | — | — | — |
| 10 | — | 0·13 | — | — | — | — | — | — | 17·0 | — | — |
| 11 | 7·5 | 0·11 | — | — | — | — | — | — | — | — | — |
| 12 | 156 | — | — | — | — | — | — | — | 40·5 | — | — |
| 13 | 2450 | — | — | — | — | — | — | — | — | — | — |
| 14 | — | — | 3184 | 37·1 | 1·3 | 3·5 | 1·9 | 3·4 | 58·0 | — | — |
| 15 | — | — | — | — | — | — | — | — | — | — | — |
| 16 | 1500 | 0·17 | — | — | — | — | — | — | 5450 | 5200 | 250 |
| 17 | — | — | 2003 | 30·3 | 4·4 | 2·2 | 1·7 | 14·3 | — | — | — |
| 18 | — | — | — | — | — | — | — | — | 5300 | — | — |
| 19 | — | — | — | — | — | — | — | — | — | — | — |
| 20 | — | — | — | — | — | — | — | — | — | — | — |

The effect of the nitrogen metabolism on the amniotic fluid of the chick has been investigated by Fiske & Boyden; Targonski, and Kamei. As Table 145 shows, the total nitrogen concentration of the amniotic liquid rises very slowly until the 12th day, at which time it increases in prodigious strides, but, as Fiske & Boyden put it, "this merely serves to give a quantitative aspect" to the fact, discovered by Hirota and Fülleborn, that in the last half of the 2nd week of incubation a communication is established between the albumen-sac and the amnion at the site of the sero-amniotic junction. As the data of Targonski and of Kamei in Table 145 show, this large increase of nitrogen is almost entirely due to protein. As for the uric acid, its concentration does not increase proportionately to the growth of the

embryo, as is the case in the allantoic liquid, but it remains constant throughout. No communication between cloaca and amniotic fluid exists till later—after the 17th day, according to Gasser. Kamei's figures for total non-protein nitrogen basic and non-basic by phosphotungstic acid remain difficult to interpret, partly owing to their small number, but it is interesting that he found a definite increase in the ammonia and urea concentration of the amniotic fluid. Such easily soluble and diffusible molecules would be expected to penetrate the walls of the allantois, and to turn up elsewhere.

Targonski has calculated the ratios Nitrogen in embryo/Nitrogen in amniotic liquid and Nitrogen in amniotic liquid/Nitrogen in allantoic liquid. Both these are plotted in Fig. 334.

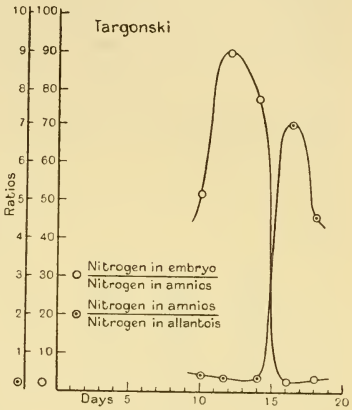



Fig. 334.

The first ratio is high and rising, for the denominator is remaining constant and the numerator is steadily increasing, but a maximum occurs on the 12th day, owing to the sudden inrush of protein from the albumen-sac into the amnios through the sero-amniotic connection, and afterwards a fall takes place. The same relations hold inversely for the amniotic and allantoic liquids, for the nitrogen of the latter is at first abundant compared to that of the former, but, after the events of the 12th day, this is no longer the case. Targonski's curves illustrate the effects of the opening of the sero-amniotic junction.

## 9·7. The Origin of Protective Syntheses

The only other matter which must be considered under the heading of the protein metabolism of the bird's egg is the synthesis of ornithuric acid by the embryo. It is obviously a matter of great interest to determine the time in ontogenesis at which the embryo becomes able to carry out those chemical protective syntheses which are so interesting a feature of the metabolism of the adult. Takahashi, who

first attacked this problem, estimated the sulphates in the allantoic liquid, and found, as will be described in Section 12·7, that at least as early as the 9th day ethereal sulphates were accumulating in the embryonic excreta, showing a detoxication of phenols by synthesis with sulphuric acid. But his work on ornithuric acid was more remarkable. As is well known, the metabolism of the bird reacts to the presence of benzoic acid by combining it with ornithine (diamino-valerianic acid) and excreting it in that form—a mechanism doubtless developed because of the vegetarian diet, and its accompanying abundance of unbreakable benzene rings. In order, therefore, to discover at what stage in development the chick could first bring about this synthesis, Takahashi injected small amounts of sodium

Table 146.

| | | | | Amount of benzoic acid found afterwards | | | Amount of ornithuric acid found afterwards | | |
|---|---|---|---|---|---|---|---|---|---|
| Day | No. of eggs taken for experiment | No. of eggs developing normally and used for estimations | Total amount of benzoic acid injected (gm.) | Allantois | Yolk and white | Embryo and amnios | Allantois | Yolk and white | Embryo and amnios |
| 0 | All injections made | | | | | | | | |
| 9 | 1014 | 842 | 3·537 | 0·0614 | 0·1372 | 0·0 | 0·0 | 0·0 | 0·0 |
| 14 | 2094 | 1411 | 5·928 | 0·0 | 0·0 | 0·0 | 0·4541 | 0·0 | 0·0 |
| 18 | 1292 | 774 | 3·251 | 0·0 | 0·0 | 0·0 | 0·5964 | 0·0 | 0·0 |

The ornithuric acid was identified by solubilities, melting-point and complete elementary analysis. In control normal embryos no ornithuric acid was ever found in any part of the egg.

benzoate into the egg-white before incubation, and then worked up the various component parts of the egg for benzoic and ornithuric acid. His results, which are given in Table 146, were remarkable, partly because of the large number of eggs used, amounting to some thousands, each one of which received by injection 5 mgm. of sodium benzoate in 10 per cent. solution. The results showed definitely that no ornithuric acid was formed up to the 9th day, and that the benzoic acid was then being excreted unchanged, but that on the 14th day, on the contrary, ornithuric acid was undoubtedly being manufactured. It would probably be legitimate to conclude from this that the power to conjoin benzoic acid and ornithine is not always present in the chick embryo, but arises at a definite stage in its development.

## 9·8. Protein Metabolism of Reptilian Eggs

A group of Japanese workers has studied the development of the marine turtle, *Thalassochelys corticata*, which lays eggs the size and appearance of ping-pong balls in the warm littoral sand. Dealing first with the end products of protein metabolism, Tomita reported a preponderance of urea over uric acid. His figures were as follows, both urea and uric acid being given as absolute amounts in milligrams per egg:

| Days | Urea | Uric acid |
|---|---|---|
| 0 | 2·0 | 0·0 |
| 15 | 3·7 | 0·17 |
| 30 | 10·0 | 0·10 |
| 45 | 24·5 | 0·15 |

They lead to a typically aquatic nitrogen utilisation (see Table 163). The preponderance of urea is curious in view of the uricotelic metabolism of the sauropsida, but the nitrogen partition of chelonian urine almost certainly does not follow the usual course in reptilia, and there is abundant evidence that the turtle egg is not a cleidoic system (see Section 6·6 and the Epilegomena).

The more general aspects of nitrogen metabolism were investigated by Nakamura, who observed a diminution in the total nitrogen of the eggs from 592 to 506 mgm. per egg. This is very interesting in view of Tomita's findings, for the egg of this turtle thus not only absorbs water from its environment but also gives off end-products of nitrogen catabolism to it. Transition from ureotelic to uricotelic habit occurs, then, not between amphibia and reptiles but between *Chelonia* and *Sauria*. The following table shows the movements of the nitrogen within the egg according to Nakamura's analyses:

Table 147.

| | Milligrams nitrogen | | | | |
|---|---|---|---|---|---|
| Day | Whole egg | Egg-white | Egg-yolk | Amniotic and allantoic liquids | Embryo |
| 0 | 592 | 41·2 | 548 | 3 | — |
| 16 | 586 | 17·8 | 544 | 24 | — |
| 30 | 549 | 12·2 | 501 | 25 | 10 |
| 45 | 506 | — | 119 | 64 | 323 |

| | Grams weight of the fractions | | | |
|---|---|---|---|---|
| Day | Egg-white | Egg-yolk | Amniotic and allantoic liquids | Embryo |
| 0 | 18·7 | 11·4 | 3·3 | — |
| 16 | 10·5 | 10·8 | 12·5 | — |
| 30 | 5·8 | 10·4 | 15·1 | 1·1 |
| 45 | — | 2·6 | 12·7 | 17·5 |

Table 147 shows clearly the passage of nitrogen from raw materials into embryo and also the fact that, just as with the bird, the egg-white is used up before the yolk. Nakamura estimated the non-protein nitrogen in the various parts of the egg during development but it did not vary much, remaining always at from 14 to 25 mgm. per cent., and showing only a slight rising tendency.

Another member of the group, Sendju, studied the behaviour of the amino-acids with the following interesting results:

Table 148.

Total amino-acids in milligrams per egg

| Days | Trypto-phane | Tyro-sine | Cystine | Arginine | Histi-dine | Lysine | Purine bases |
|---|---|---|---|---|---|---|---|
| 0 | 55 | 173 | 59 | 208 | 33 | 212 | 0·4 |
| 15 | 46 | 156 | 52 | 195 | 35 | 201 | 0·7 |
| 37 | 39 | 135 | 41 | 193 | 45 | 187 | 2·7 |
| 45 | 38 | 115 | 31 | 176 | 47 | 201 | 3·3 |
| Hatched | 36 | 115 | 27 | 181 | 48 | 196 | 3·6 |

From Table 148 and Figs. 295 to 298 it is clear that the turtle's egg has only half as much tryptophane, tyrosine and lysine as the hen's egg, and still less cystine and histidine. The loss during development seems to bear mainly on the tryptophane, tyrosine, cystine and arginine, i.e. precisely those amino-acids in which definite diminutions had been found by Sendju to occur during avian ontogenesis. In both the turtle and the hen, the lysine remains constant, and the purine bases are synthesised although it may be questioned whether Sendju's figures for the latter are not much too low. Tomita found the following distribution as between white and yolk in the turtle's egg:

| | Milligrams % (fresh egg) wet weight | |
|---|---|---|
| | White | Yolk |
| Tryptophane ... ... ... | 0 | 55 |
| Tyrosine ... ... ... | 103 | 153 |
| Cystine ... ... ... | 123 | 201 |
| Arginine ... ... ... | 32 | 1089 |
| Histidine ... ... ... | 9 | 201 |
| Lysine ... ... ... ... | 9 | 1354 |
| Purine bases ... ... ... | 0 | 3 (?) |

## 9·9. Protein Metabolism of Amphibian Eggs

Fauré-Fremiet & Dragoiu in their analysis of the frog's egg and the hatched tadpole, observed a definite diminution of protein. The figures were as follows:

Table 149.

| | | Protein | | |
|---|---|---|---|---|
| | | % wet weight | % dry weight | Milligrams absolute |
| Terroine & Barthélemy | Zero hour | 27·9 | — | — |
| Fauré-Fremiet & Dragoiu | Zero hour | 26·5 | 61·0 | 1·1696 |
| Fauré-Fremiet & Dragoiu | Hatching | 23·2 | — | 1·0459 |
| Therefore lost during this time | | 3·3 | — | 0·1237 |
| Fauré-Fremiet & Dragoiu | Loss of yolk-sac | | — | 0·9000 |
| Therefore lost during this time | | — | — | 0·146 |
| Lost during the whole time | | — | — | 0·270 |
| | | | | (I.c. 23·1 % of protein originally present) |

Evidently a good deal of the protein material with which the egg begins is transformed into the protein of the larva, while a smaller amount is combusted. Unfortunately Fauré-Fremiet & Dragoiu did not estimate the protein present at the last stage of all, and relied on approximative assessment, but still their data give a general idea of the process as a whole.

The other investigators who have studied the chemistry of the developing tadpole have for the most part paid no attention to its protein metabolism. But a notable exception is afforded by the interesting paper of Bialascewicz & Mincóvna which appeared in 1921. These workers estimated both the nitrogen contained in the embryos at different stages and the nitrogen excreted into the circumambient water on each day. Bialascewicz & Mincóvna first established the fact that some dry weight is lost by the frog embryo—an important point, for earlier workers had confused the issue by always expressing results in percentages. The data are shown in Fig. 335, in addition to some others collected by Bialascewicz himself in another paper, and by Galloway. Using different temperatures, and consequently different hatching times (68, 78, 90, 96, 100, 120 and 172 hours), Bialascewicz & Mincóvna found that the loss of dry solid was always practically identical at

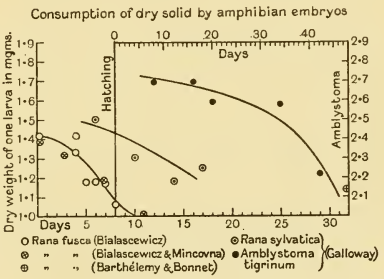



Fig. 335.

0·076 mgm., or 5·6 per cent. of the initial dry weight. They next estimated the nitrogen in the egg at the beginning and in the hatched tadpole at the end, using the micromethods of Pilch and Bang, and obtaining the following averages:

| | Milligrams nitrogen per 100 embryos | | |
|---|---|---|---|
| | Bialascewicz & Mincóvna | Barthélemy & Bonnet (for comparison) | |
| Just after fertilisation ... ... ... | 13·14 | 18·7 (without mucin) | 24·1 (with mucin) |
| At hatching ... ... ... ... | 11·95 | — | |
| At time of disappearance of external gills | — | 13·9 | |
| Loss | 1·19 | 4·8 | |

One hundred embryos during their pre-natal life, then, lost 1·19 mgm. of nitrogen, or 9·1 per cent. of the initial quantity provided. After hatching this decomposition of protein still goes on, as Fig. 336, taken from Bialascewicz & Mincóvna's figures, shows. Turning now to the determination of the end products, the total nitrogen excreted by the embryos into the surrounding water was estimated, and related to a standard number of embryos in 24-hour periods. This led to the striking curve in Fig. 337, which shows the relative intensity of nitrogen excretion from frog embryos, and as it is an intensity graph it can be compared directly with that for the chick as given in Fig. 325. To express the amphibian production of nitrogen in milligrams per cent. dry weight of embryo is not possible, as the embryo cannot be dissected away from the yolk. We can only tell what 100 mgm. dry weight of frog embryo-plus-yolk excretes per day, but, as the total change of dry weight throughout development is not more than 6 per cent., there is no necessity to turn the excretion curve into terms of dry weight, and it is sufficient to have it in terms of a constant number of embryos. It is obvious that, if we could express it in terms of "live" dry weight, the peak would be much more in evidence than it is, for the "live" dry weight is continually increasing, and the "dead" or yolk dry weight is continually decreasing—therefore the descent after the 125th hour would be emphasised considerably. It may be said, then, that in the frog, as well as in the chick, there exists a period towards the middle of embryonic development at which more protein is combusted than at any other time. The observation of this peak in a bird and an amphibian would suggest that the phenomenon is a general one.

It was further found that the excretion was almost equally divided between urea and ammonia, as can be seen by the diagram at the top of Fig. 337. Bialascewicz & Mincóvna noted that, while the loss in dry weight up to hatching was 0·076 mgm. per embryo, the loss in protein nitrogen was 0·012 mgm. per embryo or of protein 0·075 mgm.;—they were inclined to hold, therefore, that before hatching only minimal amounts of fat and carbohydrate could be combusted. Other investigators, however, have not agreed with this conclusion (see p. 1175). The 0·075 mgm. of protein disappearing would roughly

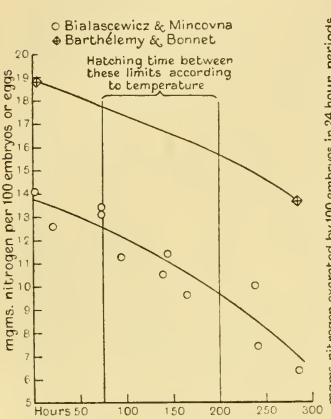



Fig. 336.

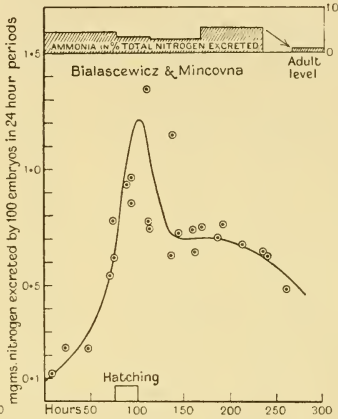



Fig. 337.

correspond with the 0·0039 c.c. of oxygen which was found by Bialascéwicz & Bledovski to be taken up by one from embryo between fertilisation and hatching.

Barthélemy & Bonnet found, as has been stated above, that one egg of *Rana temporaria* contained 0·187 mgm. nitrogen without its mucilaginous envelope, and 0·241 with it, while at hatching one embryo contained 0·139 mgm. nitrogen. This was a loss of 25·7 per cent. analogous to the loss of 23·1 per cent. found by Fauré-Fremiet & Dragoiu up to the end of the free-swimming yolk-provided stage. But the point on which Barthélemy & Bonnet laid stress was that, no matter what the temperature and therefore the rapidity of

development was, this value remained unaltered. In other words, it is useless to attempt to alter the total amount of nitrogen wastage associated with the making of one frog embryo by varying the temperature, for all that will change will be the rate of the whole process[1]. The following figures illustrate this:

| Temperature | Nitrogen excreted in % of the initial store | Days taken till disappearance of external gills |
|---|---|---|
| 8 | 26·5 | 30 |
| 10 | 28·9 | 22 |
| 14 | 25·1 | 20 |
| 21 | 25·7 | 8 |

Hibbard has described in the anuran, *Discoglossus pictus*, the accumulation and excretion of little superficial vacuoles in the epithelial cells of the pre-hatching stages. These she regards as possibly the mechanism of excretion of end-products prior to the formation of the kidneys.

With the work of Gortner we pass to another aspect of the attack on the protein metabolism of the amphibian embryo, although he was concerned with an icthyoid urodele, *Cryptobranchus allegheniensis* (the American giant salamander or "hell-bender"), and not with the anura. In many respects his findings differ considerably from those of the workers who have dealt with the latter material, and, as *Cryptobranchus* eggs are very difficult to obtain, it is not likely that he will be contradicted or confirmed very soon. Nevertheless, further work would be desirable on this or a related animal, for Gortner could find very little nitrogenous waste, and it is difficult to believe that his figures for this are correct. He approached the subject from the same angle as Plimmer & Lowndes, and, extracting the eggs and hatched larvae first with ether and then with alcohol, he divided them into what was soluble in ether and alcohol and what was not, which latter residue he called the "protein" fraction. Then he determined the different kinds of nitrogen in each. Table 150 shows his results.

Taking the protein first, Gortner found that 100 eggs had 575·4 mgm. of protein nitrogen and 100 hatched embryos had 568·8, a loss of 6·6 mgm., which he did not regard as significant. The character of the protein in the eggs certainly differed from that in the embryos at hatching, the main change being that, in the latter, the mono-amino fraction had decreased and the di-amino fraction had slightly increased. This finding agrees with the conclusions of Plimmer & Lowndes on the hen's egg, and not with those of Russo. Slight rises

[1] Cf. Section 6·10.

were noticeable in the ammonia nitrogen, the humin nitrogen, and in the non-amino nitrogen in the phosphotungstic filtrate. These are unexplained. Within the di-amino fraction the arginine, lysine and cystine rose, while the histidine fell, some of which changes agree with those found for the chick, and some of which do not.

Table 150. *Gortner's figures for* Cryptobranchus allegheniensis.

| | % of the total nitrogen | | |
|---|---|---|---|
| Ether-soluble fraction | Undeveloped eggs | Hatching larvae | |
| Ammonia nitrogen ... ... | 0·048 | 0·108 | Rise |
| Humin nitrogen ... ... ... | 0·191 | 0·383 | Rise |
| Basic (di-amino) nitrogen ... | 0·048 | 0·048 | No change |
| Non-basic (mono-amino) nitrogen | 0·114 | 0·239 | Rise |
| Total ... ... | 0·401 | 0·778 | Rise |
| Alcohol-soluble fraction (ether-insoluble) | | | |
| Ammonia nitrogen ... ... | 0·024 | 0·024 | No change |
| Humin nitrogen ... ... ... | 0·287 | 0·407 | Rise |
| Basic (di-amino) nitrogen ... | 0·654 | 0·838 | Rise |
| Non-basic (mono-amino) nitrogen | 0·231 | 0·635 | Rise |
| Total ... ... | 1·196 | 1·904 | Rise |
| Protein fraction | | | |
| Ammonia nitrogen ... ... | 9·956 | 10·21 | Rise |
| Humin nitrogen ... ... ... | 2·250 | 2·324 | Slight rise |
| Di-amino nitrogen total ... ... | 19·56 | 19·38 | Slight fall |
| Arginine nitrogen ... ... | 13·56 | 13·90 | Slight rise |
| Histidine nitrogen ... ... | 5·63 | 4·93 | Fall |
| Lysine nitrogen ... ... ... | 10·16 | 10·27 | Slight rise |
| Part of cystine nitrogen ... | 0·213 | 0·282 | Slight rise |
| Mono-amino nitrogen total ... | 53·73 | 51·92 | Fall |
| Non-amino nitrogen in filtration ... | 2·76 | 2·686 | Rise |
| | 88·256 | 86·52 | |

Gortner unfortunately did not make analyses for urea and uric acid by direct methods, and their presence or absence has therefore to be discussed on the basis of their probable solubilities under his special conditions. Although it is always stated that uric acid is insoluble in ether and alcohol, and that urea is insoluble in ether, Gortner affirmed that in continuous extraction this was not the case, and that appreciable quantities of these substances would pass out into the extracting liquid. Although he did not fully prove this point, his main conclusion rests upon it. If the urea and uric acid were not passing out into the ether and alcohol, they were remaining behind masked in the "protein" fraction, so that it would not be surprising if by these methods no urea production could be demonstrated. As Gortner hydrolysed all his fractions, the urea and uric acid nitrogen

would, he thought, appear as ammonia nitrogen. This, as Table 150 shows, rises in two fractions, from 0·048 to 0·108 per cent. of the total nitrogen in the ether-soluble fraction, and from 9·956 to 10·21 per cent. in the protein fraction, and remains constant in one, the alcohol-soluble fraction. The total gain in ammonia nitrogen, namely, 0·314 per cent., would certainly not counterbalance the loss in mono-amino-acids minus the gain in di-amino-acids, i.e. 1·63 per cent. However, Gortner's conclusion that "no appreciable amount of urea or uric acid is formed during embryonic growth in *Cryptobranchus*" does not follow, for the urea nitrogen may well have got mislaid among the numerous other fractions, e.g. the non-basic nitrogen of the alcohol-soluble fraction, which, in fact, does increase by 0·304 per cent. The question cannot be regarded as settled until the same material is re-investigated, using direct and modern methods for ammonia, urea and uric acid. The gain in nitrogen of the ether-soluble fraction Gortner interpreted as being due to a synthesis of lecithin such as Tichomirov observed in the eggs of the silkworm. The gain in nitrogen of the alcohol-soluble fraction he interpreted as being due to the synthesis of purine and pyrimidine bases.

As already mentioned, the loss of protein nitrogen was, per 100 eggs, 6·6 mgm., or 1·15 per cent. of the initial value, but the protein itself, measured directly, fell from 4026 to 3828 mgm., i.e. 198 mgm., or 4·92 per cent. Thus the protein at the end of development had a distinctly higher nitrogen content than that at the beginning, being 14·86 instead of 14·3 per cent., and it is possible, as will be shown later (see p. 1178), that the missing material contributed to the production of some synthesised fat. It must be remembered that it is not correct to regard the hatching stage in amphibia (for *Cryptobranchus* see B. Smith) as the end of embryonic development, for a considerable period subsequently elapses before the yolk-sac disappears, and before the complete larval form is attained. As regards the question whether any nitrogenous waste products are excreted into the water while the embryo is yet in the egg, Gortner found the total nitrogen to be practically identical at the beginning and at the end, being 584·9 mgm. and 584·5 mgm. per 100 embryos. It is probable, then, that in *Cryptobranchus* little or no waste nitrogen is excreted into the surrounding water before hatching, when what has accumulated is discharged all at once, after which the animal can get rid of its end-products without hindrance. The loss in dry weight amounted to

96·9 mgm. (5825 to 5728) per 100 eggs, and reason will be given later for supposing that this is largely, perhaps preponderantly, due to the combustion of carbohydrate.

## 9·10. Protein Metabolism in Teleostean Ontogeny

Gortner applied the same methods to the study of the protein metabolism of the brook-trout's egg (*Savelinus fontinalis*). This was more satisfactory than his study of the salamander's egg, among other reasons because he pursued its development to completion, i.e. until the larvae had lost their yolk-sacs, and were ready to take food. The figures for total nitrogen were as follows:

| Days | Total nitrogen in 400 eggs (mgm.) | Dry weight of 400 eggs (gm.) |
|---|---|---|
| 0 | 873·5 | 7·5404 |
| 21 | 890·8 | 7·4920 |
| 35 | 860·3 | 7·5287 |
| 51 (hatching) | 823·8 | 7·0668 |
| 72 (end of yolk-sac period) | 681·8 | 5·6295 |
| | | Total weight lost |
| Total nitrogen lost | 191·7 | 1·9109 |
| | (or 21·9 % of initial value) | (or 25·3 % of initial value) |

Gortner attached no importance to the gain of 20 mgm. recorded for the second sample, or to the loss of 30 mgm. recorded for the third. He preferred to average the first three readings, and to say that, before hatching, 400 eggs of the trout have a constant figure for total nitrogen at 874·8 mgm. The difference between this figure and that first obtained after hatching, namely, 51 mgm., he put down to the loss of the egg-membranes, but it is more than probable that the greater part of it consisted of nitrogenous end products which had been retained in the eggs, and were now liberated. Certainly the consumption of protein after hatching during the last third of development was considerable. We can make a rough calculation to see how much of the 51 mgm. was egg-membrane protein, for Kronfeld & Scheminzki noted that the membranes of the trout egg were 8·25 per cent. of the total dry weight, i.e. 582 mgm. in this case at hatching, or, taking its nitrogen content at 14·5 per cent. and assuming that the membrane is at least 50 per cent. keratin, 42 mgm. of nitrogen. But this is a high estimate, for it is known that the egg-membranes of fishes become diaphanous and

thin towards hatching, and the operation of the hatching enzyme (see Section 24·2) will not be without a powerful effect. The amount of nitrogen accounted for by the membrane might therefore leave some 20 mgm. for the urea nitrogen, which would thus be 2·3 per cent. of the initial nitrogen content. But such a calculation is beset with the difficulties due to our ignorance, and no great emphasis can be laid upon it.

Table 151. *Gortner's figures for* Savelinus fontinalis.

| | % of total nitrogen | | | | |
|---|---|---|---|---|---|
| Days ... | 0 | 21 | 35 | 51* | 72† |
| Ammonia nitrogen ... | 7·56 | 8·80 | 7·86 | 8·78 | 9·19 |
| Humin nitrogen ... | 1·82 | 2·26 | 2·32 | 2·70 | 2·73 |
| Di-amino fraction | | | | | |
| Total ... ... | 28·60 | 29·34 | 32·63 | 29·78 | 34·06 |
| Arginine nitrogen ... | 11·09 | 11·60 | 13·32 | 12·26 | 12·52 |
| Cystine nitrogen ... | 0·34 | 0·40 | 0·52 | 0·40 | 0·50 |
| Histidine nitrogen ... | 8·83 | 8·79 | 8·04 | 9·20 | 12·01 |
| Lysine nitrogen ... | 8·33 | 8·57 | 10·66 | 7·91 | 9·02 |
| Mono-amino fraction | | | | | |
| Total ... ... | 61·55 | 59·00 | 56·87 | 55·85 | 54·33 |
| Non-amino nitrogen | 3·90 | 4·27 | 4·09 | 3·85 | 3·90 |

| | Loss or gain (%) | | | |
|---|---|---|---|---|
| | To hatching | To end of yolk-sac period | Total period | |
| Ammonia nitrogen ... | +1·22 | +0·41 | +1·63 | |
| Humin nitrogen ... | +0·98 | +0·03 | +1·01 | +8·10 |
| Di-amino nitrogen ... | +1·18 | +4·28 | +5·46 | |
| Mono-amino nitrogen | −5·70 | −1·52 | −7·22 | |

* Hatching. † End of yolk-sac period.

Gortner's data for the distribution of nitrogen are seen in Table 151. As he took no trouble to prepare the pure protein of the eggs and embryos, it is not easy to criticise the results, but there is an un-

Table 152. *Gortner's figures for* Savelinus fontinalis.

| | % of total nitrogen | |
|---|---|---|
| | Protein at zero hour | Protein in combusted fraction |
| Ammonia nitrogen ... ... | 7·56 | 1·77 |
| Di-amino nitrogen ... ... | 28·60 | 9·17 |
| Mono-amino nitrogen ... ... | 61·55 | 87·14 |

mistakable trend from the mono-amino to the di-amino nitrogen, exactly as would be the case if there was a preferential utilisation of the former for combustion purposes. This appears well in Table 152

where the composition of the lost nitrogen is shown. Here again is a correspondence with Plimmer & Lowndes. The ammonia and humin nitrogen also rise.

In this connection reference must be made to the paper of Tangl & Farkas on the trout egg, which has been already discussed on p. 955. In the course of their calorimetric study they obtained the following figures:

| | Milligrams per 100 individuals | | |
|---|---|---|---|
| | Undeveloped eggs | Hatched fry | Gain or loss |
| Wet weight ... ... | 8850 | 8330 | −520 |
| Dry weight ... ... | 2990 | 2915 | −75 |
| Fatty acids ... ... | 639 | 663 | +24 |
| Nitrogen ... ... | 359 | 357 | −2 |
| Carbon ... ... | 1672 | 1635 | −37 |

Tangl & Farkas did not regard the loss of nitrogen as significant, but there was certainly a gain in fatty acids. This phenomenon will receive some consideration in Section 11·8; all that need be said here is that this demonstration of the fixity of the total nitrogen does not imply that no protein had been catabolised, as the urea or ammonia would be included in the total nitrogen.

Gortner determined the phenol content of the phosphotungstic filtrate, and observed a loss of 29 per cent. throughout the whole period, principally after hatching, which may or may not have been tyrosine. As stated above, the 400 embryos lose throughout the whole period 1911 mgm., of which 1190 mgm. is protein, so Gortner concluded that 62·7 per cent. of the total food-stuff catabolised must come from the proteins, and 37·2 per cent. from other substances. (See Table 126.)

Pearse has also studied the eggs of fresh-water fishes, particularly the brook-trout. In his paper of 1925 he stated that the nitrogen diminishes from the 1st day of development till after the end of the yolk-sac period, but the published details are meagre. Hayes states that there is no diminution of the total nitrogen before hatching in the egg of the Atlantic salmon (*Salmo salar*) but that there is in that of the lumpsucker (*Cyclopterus lumpus*). Levene's experiments on the cod's egg (*Gadus morrhua*) were not unlike those of Gortner on the trout. The following table shows that the eggs as a whole lose water as they develop, and that there is a distinct loss of nitrogen, although the figures are erratic. The data for basic and non-basic nitrogen and protein nitrogen are so uneven, rising and falling in leaps between

each set of readings, that it is impossible to interpret them, and useless to present them.

Table 153.

| Days after fertilisation | Water (%) | Ash (% dry weight) | Nitrogen (% dry weight) |
|---|---|---|---|
| 0 | 94·66 | 10·09 | 10·90 |
| 1 | 94·80 | 17·17 | 9·96 |
| 11 | 92·02 | 17·55 | 11·22 |
| 20 | 93·59 | 19·66 | 9·52 |
| | | Loss ... ... | 1·38 |

A related form which has been investigated is the egg of the plaice, *Pleuronectes platessa*, the nitrogen content of which was determined by Dakin & Dakin in 1925. Their table,

Table 154.

| | 2000 eggs (weights in milligrams) | |
|---|---|---|
| | fertilisation | hatching |
| Wet weight ... ... ... | 6720 | 6638 |
| Dry weight ... ... ... | 479 | 418 |
| Water ... ... ... ... | 6251 | 6220 |
| Fatty acids ... ... ... | 6 | 21 |
| Protein ... ... ... ... | 426 | 348 |
| Protein nitrogen ... ... | 68 | 56 |

shows that, during the period preceding hatching, 78 mgm. of protein are lost by this quantity of eggs, or 18·3 per cent. of the original amount. In this egg, therefore, the membranes must clearly resemble those of the eggs of the anura rather than those of the eggs of the salmonidae, in letting the products of protein combustion pass out to the exterior. It is also obvious from Table 154 that the loss of protein almost wholly accounts for the loss in dry weight, so that between 80 and 95 per cent. of the total material catabolised must be, in the case of the plaice, protein. The oxygen consumption (90 mgm. per 2000 eggs), as found by Dakin & Dakin, nearly, but not quite, equals that calculated from the lost protein, assuming that it was all combusted. At the same time there is the 15 mgm. increase in fat to be remembered.

## 9·11. Protein Metabolism in Selachian Ontogeny

With this we pass to the elasmobranch fishes. Little has been done on the protein metabolism of their embryos, but it could have been predicted beforehand that in this they would differ from other fishes.

In the adult elasmobranch, the urea produced by protein breakdown is retained within the circulation, causing an extraordinarily high blood urea and counterbalancing the osmotic pressure of the saline external medium. This retention of urea as an osmotic device has already been discussed in Section 1·13. As early as 1834 John Davy noted the presence of urea in the uterine cavity of *Squalus squatina*, a selachian, but did not succeed in finding any in the uterus of *Torpedo marmorata*. He never detected uric acid in these fishes. Parker and Parker & Liversidge also found abundance of urea, but no protein or uric acid, in the "pseudamniotic liquid" (i.e. the perivitelline liquid within the egg-cases of the ovoviviparous *Mustelus antarcticus*, after the disappearance of most of the yolk).

Whence comes all this urea? In 1890 von Schröder extirpated the livers of a number of fishes (*Scyllium canicula*) and observed only a small reduction of the urea content of the muscles (1950 mgm. per cent. before and 1860 mgm. per cent. afterwards). The liver can therefore not be the main source, and probably all the tissues have the power of forming urea from the amino-groups in the food. Arginase appears to be found in great quantities in elasmobranchs; thus Hunter & Dauphinee found the following typical figures:

| | Arginase units | |
|---|---|---|
| | Liver | Kidney |
| Selachian (*Squalus sucklii*) ... ... | 319 | 31 |
| Teleostean (*Sebastodes maliger*) ... | 29 | 4 |

The arginase of the dogfish liver was twice as active as that from the most active teleostean liver (in the herring, *Clupea pallasii*) and forty times as active as that from the feeblest (in the tommy-cod, *Hexagrammos stelleri*). It is probable, however, that the contribution of urea made by arginase to the total urea content of the elasmobranch cells would not be large. Hunter & Dauphinee made the interesting observation that the undeveloped eggs of *Squalus sucklii* contained notable amounts of urea, but no arginase, while both were present in an embryo of 20·5 cm. (see also pp. 1077, 1142 and 1312).

It may be added that Baglioni found that the selachian heart could not beat properly unless a certain amount of urea was contained in the perfusion fluid.

The excretion of urea in selachians does not appear to take place wholly through the kidneys. Denis found that only 20 to 50 mgm

were excreted through the kidneys of an adult dogfish per kilo per day. The gills have been found by Duval & Portier to be absolutely impermeable to urea. But van Slyke & White found that large amounts of urea were contained in the bile (up to 72·3 per cent. of the total biliary nitrogen), so that the intestinal tract is probably concerned together with the liver cells in regulating the urea-content. The kidney certainly does not seem to do much regulation, as Denis found the blood urea to be uninfluenced by experimental nephritis induced by uranium nitrate or potassium chromate. Another mode of elimination of urea from the elasmobranch body is through the peritoneal pores, which Smith believes to have an excretory function, for the peritoneal fluid of a dogfish contains 680 mgm. per cent.

More recently, Needham & Needham made an investigation of urea production in the embryos of *Scyllium canicula* and *Pristiurus melanostoma*. We first of all carried out a series of experiments to ascertain whether the dogfish egg was a closed system, and, by allowing eggs to remain in comparatively restricted amounts of sea water, we found that not more than traces of urea are lost to the exterior, no matter whether the embryo be less than 1 cm. long, or more than 7, i.e. nearly ready to hatch. Rather complicated controls were here necessary as the diatoms in sea water destroy urea, a fact insufficiently taken into account by earlier workers. By constructing osmometers of the thick horny egg-shells, we further found that, although they themselves were permeable to urea in the outward direction yet the slimy coat on the inside effectually made them impermeable. But the fact that the egg was a closed system as regards nitrogenous end products led to a paradox, for, as is well known, the egg-cases of the dogfish possess from the beginning four slits at one end, which, at first plugged with albumen, open to the sea water about two-thirds of the way through development. It is even probable that a current of sea water may pass through them, introduced by the waving of the embryo's external gills. Why, then, does not the urea escape? We found the answer when we estimated the urea in yolk, egg-white, and embryo separately:

| | Urea (mgm.) |
|---|---|
| 0·5 cm. embryo and yolk ... ... | 8·32 |
| Corresponding white ... ... | 0·165 |
| 3·0 cm. embryo and yolk ... ... | 11·59 |
| Corresponding white ... ... | 0·03 |

Evidently the dogfish embryo excretes its urea into its yolk, and so retains it for its osmotic purposes. This peculiarity, then, made it possible to regard the egg as a closed system, and to estimate the amounts of urea produced by the embryo at different ages, the results of which are shown in Fig. 338. By the end of development some 15 mgm. of urea are present, but, as we do not yet know the exact amount of protein nitrogen present at the beginning, we cannot say what percentage this is.

Unfortunately, no good series of weighings exists for *Scyllium* embryos, but we know from the work of Fulton and Kearney that the length of fish embryos increases at first far more rapidly than the weight. Now this evidently implies that when the points are plotted against weight, the slight concavity towards the abscissa, shown in Fig. 338, will be greatly accentuated, because equal increments of length mean much greater increments of weight in the later stages than in the earlier. Plotted against weight, then, the urea-content curve would rise sharply to a certain point and then very slowly. Accordingly when the urea produced by the embryo is referred to unit weight of embryo, a peaked curve would result.

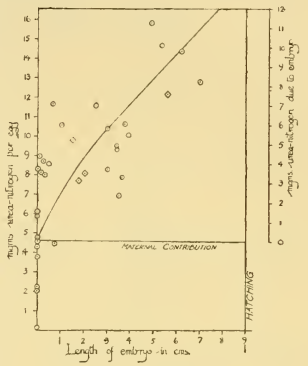



Fig. 338.

This expectation we found to be fulfilled as far as possible when we used as weight data the figures of Kearney for *Mustelus canis*. We laid no emphasis on the result though it will be admitted that the shape of the ascending weight/age curve for *Mustelus* probably does not differ much from that of the related *Scyllium*. As the last column of the following figures demonstrates, a descending curve is obtained over the range covered by Kearney.

The excretion of urea into its yolk by the elasmobranch embryo may perhaps throw light on some of the morphological results of Borcea, who studied in detail the development of the urinogenital system in these fishes.

Summing up what we know of the protein catabolism of the fish

embryo, it may be said that it certainly uses more protein as an energy source than terrestrial embryos do, whether this be expressed in terms of the initial store of protein or of the total amount of material catabolised. Such a rule would be expected from the composition of the fish egg, which, as Greene said, has on an average 27 per cent. of protein in its yolk as against the 16 per cent. of protein present in the yolk of the chick.

| Length in cm. (*Scyllium*) | Urea-nitrogen milligrams per embryo (embryo's contribution only) | Kearney's corresponding weights in milligrams (*Mustelus*) | Milligrams urea-nitrogen produced per 100 milligrams wet weight |
|---|---|---|---|
| 0 | 0 | — | — |
| 0·5 | 1·35 | — | — |
| 1·0 | 2·4 | — | — |
| 1·5 | 3·4 | — | — |
| 2·0 | 4·3 | — | — |
| 2·5 | 5·1 | — | — |
| 3·0 | 5·8 | 110 | 5·250 |
| 3·5 | 6·4 | 200 | 3·200 |
| 4·0 | 7·1 | 300 | 2·370 |
| 4·5 | 7·7 | 400 | 1·920 |
| 5·0 | 8·4 | 550 | 1·550 |
| 5·5 | 9·0 | 730 | 1·230 |
| 6·0 | 9·6 | 920 | 1·042 |
| 6·5 | 10·3 | 1150 | 0·899 |
| 7·0 | 11·0 | 1450 | 0·760 |
| 7·5 | 11·6 | 1720 | 0·668 |
| 8·0 | 12·2 | 2050 | 0·595 |

## 9·12. Protein Metabolism of Insect, Worm and Echinoderm Eggs

Among the insects, the egg which has received the most examination from this point of view is that of the silkworm, *Bombyx mori*. Tichomirov, who studied its metabolism in 1882, observed a diminution of protein (estimated roughly by solubility) from 11·3 to 9·2 per cent. of the dry weight, i.e. 18·6 per cent. of the original material, although, as only 14·9 per cent. of the original dry weight was lost, all the protein cannot have been combusted.

In more recent times, the proteins of the silkworm egg have been investigated anew by Pigorini. He divided them into several fractions: (*A*) proteins soluble in distilled water, albumens, (*B*) proteins soluble in 7·5 per cent. sodium chloride solution, globulins, (*C*) proteins soluble in 0·5 per cent. sodium hydroxide solution, vitellin and nucleoprotein, and finally (*D*) proteins soluble in water, but incoagulable by heat, and having the property of liberating

reducing substances on acid hydrolysis, ovomucoid. Pigorini subjected the eggs of the silkworm to three successive extractions, which gave him the proteins of groups *A*, *B*, and *C*, and then by coagulation of the extracts he was able to evaluate the amount of *D*. Fig. 339 gives the results.

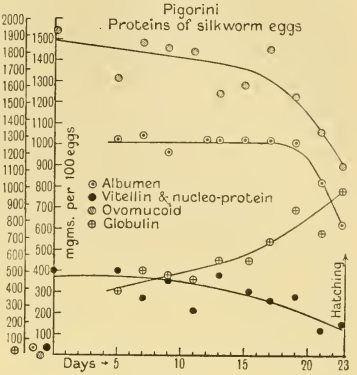


Fig. 339.

Monzini has estimated the free amino-nitrogen in the silkworm egg at different stages of development. Expressed in terms of wet weight of egg, it shows at first a not very well-defined peak, and then declines till the larvae emerge (see Fig. 340). Tirelli continued Monzini's work, but his figures are so irregular that it is impossible to plot them. Russo extended the van Slyke method to the silkworm egg, with the results shown in Table 155. According to his data the total nitrogen per cent. of the dry weight is variable, falling by about 12 per cent., and then rising by about 7 per cent. There is every reason for supposing *a priori* that no nitrogen escapes from a terrestrial egg such as that of the silkworm, for the only form in which it could do so would be gaseous ammonia, and this would be so perceptible by its odour in the silkworm establishments that it would be well known. It is more probable that Russo's estimations were not sufficiently statistical or accurate to demonstrate a constancy in the total nitrogen of the egg. Ashbel, it is true, has stated that ammonia is lost by silkworm eggs, but only on the basis of erratic positive pressures in manometric experiments.

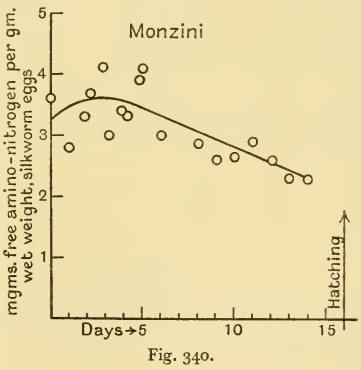


Fig. 340.

Farkas, although mainly concerned with the respiration and the calorific value of the silkworm egg at different stages, also estimated the total nitrogen in the eggs. In his first experiment a batch of 33·0 gm. of eggs (about 280 individuals) contained 1·27 gm. of total nitrogen before development, and 1·26 gm. at hatching—a constancy probably within the limits of error. His second experiment gave a different result, however, the total nitrogen falling from 1·84 gm. to 1·54 gm. for a batch of 45·87 gm. of eggs. Farkas' explanation for this was that during the last few days of development the larvae were hatching irregularly, and some were dying, so that a mass of excreta, egg-shells, and dead larvae, all saturated with condensation water, remained on the floor of the incubation chamber. In this mass, micro-organisms were doubtless decomposing the uric acid. This explanation might go some way towards explaining Russo's decline in total nitrogen. Furthermore, it was shown by Peligot for the silkworm and by Henneberg for the bee that no ammonia was given off by the eggs as they develop. Farkas consequently affirmed that there was no nitrogen loss from the eggs, and that the missing 0·27 gm. of nitrogen in his second experiment was all uric acid. Further and more accurate observations would be very desirable in this confused subject. Farkas calculated from his calorimetric measurements that 63·4 per cent. of the total material catabolised in the silkworm egg was fat, and 36·6 per cent. not fat; and for this latter fraction he calculated a specific energy of 7·31 Cals. On this basis alone (the specific energy of protein being 7·9 Cals.) he concluded that most of the 36·6 per cent. was protein. It is not sufficient evidence.

Table 155. *Russo's figures for the silkworm.*

| | Total nitrogen % dry weight | % of the total nitrogen | | | | | |
|---|---|---|---|---|---|---|---|
| | | Protein nitrogen | Non-protein nitrogen | Free amino nitrogen | Total non-protein amino nitrogen | Ammonia nitrogen | Undetermined |
| Just fertilised ... | 10·88 | 95·95 | 4·05 | 2·20 | 2·47 | 0·18 | 1·4 |
| After the diapause ... | 9·20 | 95·95 | 4·05 | 2·20 | 2·47 | 0·18 | 1·4 |
| At hatching ... | 9·91 | 92·24 | 7·76 | 0·00 | 3·93 | 0·30 | 3·53 |

More reliance, perhaps, can be placed upon Russo's figures for the distribution of nitrogen. These are interesting, for they show practically no change till the end of the diapause (as might be expected, the morphological work done till the end of that period being so

slight), but definite changes during the three weeks of active growth and differentiation in the spring. The protein nitrogen decreases by 3·71 or 3·87 per cent. of the original value, and this loss, probably all uric acid, plus the undetermined and ammonia nitrogen, almost exactly makes up the 7·76 per cent. of non-protein nitrogen present in the egg-contents at hatching. It is interesting to see the complete disappearance of the free amino-nitrogen, a result which by no means agrees with Monzini. Nor does the small loss in protein nitrogen agree with the large loss found by Pigorini, but Russo's figures are the later ones, and are certainly to be preferred.

Another insect egg which has been investigated to some extent is that of the lackey-moth or tent-caterpillar, *Malacosoma americana*. Rudolfs determined the total nitrogen in it during embryonic life, and reported simply that it rose from 11·5 per cent. of the dry weight to 14·4 per cent. at hatching. As this figure was for the whole egg-masses, including the cases, it can be only an apparent rise, due to the diminution of something else, and, as Rudolfs did not publish his data for absolute weights, his results are difficult to interpret. The fat is known to diminish in this egg, and consequently the nitrogen/fat ratio rises during embryonic life. Rudolfs also gave one analysis of the egg-contents and egg-case separately towards the end of development, but there is nothing to compare it with. Free amino-nitrogen, free ammonia, urea, tryptophane, tyrosine, etc., were all tested for and found to be present. The presence of free ammonia (3·54 mgm. per cent.) in the egg-case and of free amino-acids (0·83 mgm. per cent.) suggested to him that these were decomposing, and furnishing amino-acids to the eggs inside. Such a process had previously been thought to take place in the silkworm egg.

We know nothing about the nitrogen metabolism of nematode eggs, except that Kozmina found as much nitrogen present at the end of *Ascaris* development as at the beginning.

Our knowledge of the protein metabolism of the echinoderm egg is also in a very backward state, but Ephrussi & Rapkine have done a good deal to fill the gap. Working on the eggs of *Strongylocentrotus lividus*, they found the following figures:

| Hours from fertilisation | % of the dry weight | |
|---|---|---|
| | Nitrogen | Protein |
| 0 ... ... ... ... | 10·7 | 66·88 |
| 12 (blastulae) ... ... ... | 10·2 | 63·75 |
| 40 (plutei) ... ... ... | 9·7 | 60·62 |

It would thus appear that an appreciable amount of protein is used up during echinoderm early development. The only information we possess as to the corresponding end products is due to the work of Ashbel who estimated the ammonia produced in cultures of developing eggs, with the following results:

| | Milligrams of ammonia produced by 1 c.mm. of centrifuged eggs per hour | Cubic millimetres of oxygen used by 1 c.mm. of centrifuged eggs per hour |
|---|---|---|
| *Sphaerechinus* | | |
| Unfertilised ... ... ... | 0·289 | 263·53 |
| Fertilised ... ... ... | More | More |
| *Paracentrotus* | | |
| Time after fertilisation 6 h. 20 min. | 0·222 | 174·0 |
| ,, ,, 26 h. 0 min. | 0·287 | 525·0 |
| ,, ,, 50 h. 20 min. | 0·377 | 400·0 |

The ammonia production could be inhibited by potassium cyanide. Ashbel apparently did not look for other end products.

## 9·13. Protein Utilisation in Mammalian Embryonic Life

The examination of the nitrogen excretion of the mammalian embryo was begun by Vauquelin & Buniva in 1800, who isolated a substance resembling uric acid from the amniotic liquid of a cow embryo; to it they gave the name "amniotic acid". In 1821 Lassaigne isolated it also from the allantoic liquid of the cow, and called it "allantoic acid". Probably these early workers were dealing with impure allantoin containing traces of uric acid. Urea was not found till rather later, for Fromherz & Gugert were the first to report its presence in the amniotic liquid of man in 1827, but they were not sure that they had excluded the contamination of the maternal urine, so it was not until Rees in 1838 obtained pure amniotic liquid from a $7\frac{1}{2}$-month foetus, and found urea in it, that the production of urea by the foetus was really demonstrated. Thenceforward a large number of reports appeared, e.g. those of Wöhler; Regnauld; Picard; Majevski; Tschernov; Litzmann-Colberg; Beale; Siebert; Winckel; Grohe; Schlossberger; Mack; Vogt; Scherer, etc., in which the urea was for the most part estimated by the Liebig-Wöhler method in the amniotic liquids of man, the cow, and other animals. The data of the majority of these observers are now of little interest, for they only gave their results in terms of urea per cent. of the amniotic or allantoic liquid, and omitted to mention either the weight of

the embryo, the total volume of liquid, or some other detail essential for making any calculation or comparison. A tabular presentment of their figures is given by Prochovnik; the values vary from 27 to 420 mgm. per cent. Nevertheless, in certain instances, it is possible to calculate the amount of urea present per 100 gm. of formed embryo, thus:

Table 156.

| | Weeks | Weight of embryo (gm.) | Milligrams urea per 100 gm. embryo in allantois | Investigator |
|---|---|---|---|---|
| Sheep | 4 – 6 | 1·62 | 17·90 | Majevski |
| | 6½– 9 | 10·25 | 4·71 | |
| | 10 –12½ | 52·10 | 2·54 | |
| | 12½–18 | 207·00 | 2·72 | |
| Cow | 9 –12 | 19·50 | 8·50 | Majevski |
| | 12 –21 | 144·50 | 6·12 | |
| | 21 –27 | 342·00 | 3·96 | |
| Cat | 1 – 2 | 1·10 | 6·73 | Tschernov |
| | 2 – 4 | 12·34 | 1·87 | |
| | 4 – 6 | 40·15 | 0·48 | |
| | 6 – 8 | 98·29 | 0·34 | |

It is evident that in all cases the amount of urea produced by 100 gm. of foetus and excreted into the allantoic liquid declines as development proceeds.

It is interesting that Schondorff in 1899 concluded that the concentration of urea in the amniotic liquid in man was of much the same order as that in the blood and milk—another hint, perhaps, that such a diffusible molecule cannot be concentrated in a limited space, and so of interest in view of the discussion on p. 1133. It is evident, of course, that the urea in the amniotic liquid might come from the maternal circulation, but, on the other hand, it is certain that the foetus contributes largely to it, for as early as 1843 Prout found urea and uric acid in the fluid from the ureters of a human 8-month embryo and later Virchow; Schwarz; Dohrn, and Panzer all found urea and uric acid in the embryonic bladder. Then Wöhler found in 1846 a small uric acid calculus in a stillborn foetus, and uric acid infarcts in the kidneys of human embryos were reported by Hoogeweg; Virchow, and Salomonsen.

Gusserov in 1872 began a new line of investigation. His experiments proved that not only excretion of urine into the amniotic

liquid occurred, but also genuine discontinuous micturition, at any rate during the late stages, for he often found (69 per cent.) the bladders of newborn human infants to be full. This had already been the view of Englisch; Dohrn; Betschler; and many other obstetricians. According to Keene & Hewer who experimented with vital dyes, and to Hewer who used differential staining methods, the excretory activity of the human kidney begins as early as the 10th to the 12th week. Similarly Firket found that the cat's kidney can excrete sodium ferricyanide and ferric ammonium citrate before the glomeruli are fully differentiated. Mijsberg even has reasons for supposing that the human pronephros is functional (see also Fritschek).

Gusserov reported the presence of allantoin in the urine of pregnant women, and therefore concluded that the foetus possessed an active uricase, but this idea was exploded by Wiechovski who found no traces of it, and finally Wells & Corper discredited Cannata's assertion of the presence of uricase in the human placenta. Gusserov's original observation must have been a mistake, but he has the merit of being among the first to investigate the subject. It is not necessary here to detail the work of those investigators who have published results for urea, uric acid content, etc., of the blood of newborn infants (e.g. Martin, Ruge & Biedermann; Dohrn; Hofmeier, etc.) for the processes of parturition introduce too many doubtful factors. An interesting piece of work was later done by Feis, who found that urea injected hypodermically is a strong poison for the embryo rabbit, which cannot withstand the same degree of uraemia as the mother. In 1902 Panzer, in examining an hydropic foetus, was able to collect embryonic urine unmixed with amniotic liquid. Of specific gravity 1·008, it contained traces of protein, no glucose, keto-substances, or indican, and no creatinine. The nitrogen partition was peculiar, urea accounting for only 40 per cent. of the total nitrogen and uric acid for 15·5 per cent., the rest being in some unknown form.

Döderlein in 1890 made a detailed investigation of many aspects of the amniotic and allantoic liquids of the cow, and among his observations was a series on the non-protein nitrogen of both. Assuming that the preponderant part of this could be counted as waste nitrogen, it is easy to calculate it per 100 gm. wet weight of embryo, with the results which follow:

Table 157.

| Age of embryo in months | Wet weight of embryo | Milligrams of non-protein nitrogen in amniotic and allantoic liquids per 100 gm. embryo |
|---|---|---|
| 3 | 276 | 138 |
| 4 | 1,380 | 95 |
| | 1,800 | 67 |
| 5 | — | — |
| 6 | — | — |
| 7 | 5,123 | 78 |
| 8 | 6,690 | 47 |
| | 6,700 | 48 |
| | 8,300 | 52 |
| | 11,300 | 66 |
| 9 | 14,900 | 96 (?) |

Evidently (setting apart the last value, which is doubtful) there is here to be seen the same fall in intensity of production of nitrogenous waste which appeared above from the data of Majevski and of Tschernov—the former set indeed, fit on, as it were, at the upper end of Döderlein's. But the mere determination of non-protein nitrogen in the liquids does not carry us very far, and we find a much more thorough attack on the nitrogen excretion of the mammalian embryo in the work of Lindsay. Table 158 summarises the more important points emerging from her data. They were confined to herbivorous animals, the sheep and the cow, and, as the figures show, they were quite concordant. In both cases there was no ammonia in the foetal urine, although it was present in that of the adult. The sheep's allantoic urine contained a good proportion of allantoin, but not the cow's, and a very notable thing was the high proportion of amino-acids other than hippuric acid. Creatine and creatinine were always present, and in much the same amount, but perhaps the most extraordinary finding was that the urea of the foetal urine was very low, its percentage value being about half that of the adult urine, a deficiency made up by a large amount of unidentified nitrogen. This unidentified nitrogen confirmed the earlier work of Panzer and of Paton, Watson & Kerr, and recalls the similar assertion of Targonski in the case of the chick. Lindsay made a determined attempt to discover its nature, but without much success, although she established the fact that it was all present in the phosphotungstic acid precipitate. It was therefore probably polypeptides or di-amino-acids, and it could not have been proteoses or peptones, for the

trichloracetic acid filtrate was biuret-free. The dipeptide nitrogen, estimated by Henriques & Sorensen's method, did not account for more than a small proportion of it, nor by silver precipitation or other means was it possible to arrive at a clear idea of its nature.

Table 158. *Lindsay's figures for percentage of the total nitrogen.*

| | Ammonia | Urea | Allantoin | Amino-acids | Hippuric acid | Creatinine | Creatine | Unaccounted for |
|---|---|---|---|---|---|---|---|---|
| Sheep. Adult ... ... | 0·8 | 83·1 | 0·5 | 6·2 | 4·0 | 3·1 | 1·7 | 5·0 |
| Foetal allantoic liquid, early | | | | | | | | |
| 200–1000 gm. ... ... | None | 33·3 | 20·3 | 7·5 | 3·5 | — | — | 31·4 |
| 1000–1350 gm. ... ... | None | 15·2 | 12·4 | 10·8 | 20·9 | — | — | 42·1 |
| Full term liquid ... | None | 21·6 | 12·3 | 14·7 | 12·0 | — | — | 40·2 |
| Foetal amniotic liquid | | | | | | | | |
| 60–300 gm. ... ... | None | 64·8 | 7·0 | 5·5 | 12·0 | 0·2 | 0·2 | 18·9 |
| 300–1000 gm. ... ... | None | 63·4 | 10·3 | 7·1 | None | None | None | 21·6 |
| 1000–1350 gm. ... ... | None | 61·7 | 13·8 | 6·6 | 3·3 | 1·3 | 1·3 | 18·3 |
| Full term ... ... | None | 88·0 | 3·6 | 2·0 | 6·1 | — | — | 3·0 |
| Cow. Adult ... ... ... | 1·0 | 66·3 | 6·3 | 1·9 | 11·1 | 5·0 | 3·2 | 8·8 |
| Foetal allantoic liquid, early | | | | | | | | |
| 100–1000 gm. ... ... | None | 38·4 | 5·2 | 25·3 | 1·6 | 4·6 | 2·6 | 24·6 |
| 1000–4000 gm. ... ... | None | 22·7 | 10·2 | 25·3 | 1·6 | — | — | 40·0 |
| Full term ... ... | None | 12·6 | 18·0 | 27·1 | — | 6·6 | 6·0 | 42·2 |
| Ox. Adult ... ... ... | 1·3 | 81·2 | 0·5 | 2·4 | 3·4 | 4·3 | 3·2 | 5·4 |
| Foetal amniotic liquid | | | | | | | | |
| 100–1000 gm. ... ... | None | 63·0 | 5·2 | 12·3 | — | None | 1·9 | 19·5 |
| 1000–4000 gm. ... ... | None | 59·5 | 4·7 | 9·9 | 3·4 | None | 2·6 | 25·9 |
| 4000–6000 gm. ... ... | None | 42·8 | 16·3 | 25·4 | — | — | — | 15·3 |

One rather obvious consideration seems not to have been taken into account by Lindsay, namely, that products of the foetal protein metabolism are constantly passing through the placenta into the maternal circulation, even in the mammals with epitheliochorial placentas studied by her. There is therefore no guarantee that, when we determine the nitrogen partition in the early allantoic liquid, we are determining the nitrogen partition of the total nitrogen excretion of the embryo. Furthermore, it is obviously possible that if we knew exactly how much nitrogen the embryo was excreting and in what forms, through the placenta as well as through its own kidney, we should find such substances as amino-acids and Lindsay's unidentifiable fraction to be much less important quantitatively than would appear from her figures. And, on the other hand, there is the further possibility, though it may be remote, that the walls of the allantois

themselves contribute nitrogenous substances to the liquid within them. Lindsay's assumption, in fact, that the composition of the allantoic liquid in the early stages gives an undistorted picture of the foetal protein metabolism must be admitted only with reservations.

In this connection it is worth while anticipating the chapter on placental permeability to allude to the work which has been done on the concentration of nitrogenous end products in the maternal and foetal blood. By studying them, many investigators have hoped to discover the existence of a concentration gradient between the foetal and maternal organisms. The relevant figures are as follows (all on human blood):

Table 159.

| Urea nitrogen (mgm. %) | | Uric acid nitrogen (mgm. %) | | |
|---|---|---|---|---|
| Maternal | Foetal | Maternal | Foetal | Investigators |
| 10·5 | 10·4 | — | — | Slemons & Morriss |
| 20·1 | 20·2 | — | — | Morel & Mouriquaud |
| — | — | 3·5 | 3·5 | Slemons & Bogert |
| — | — | 3·1 | 3·1 | Kingsbury & Sedgewick |
| Less | 21·5 | — | — | Cavazzani & Levi |
| 14·8 | 15·2 | 3·9 | 3·9 | von Oettingen |
| 12·0 | 12·0 | — | — | Howe & Givens |
| No difference | | No difference | | Caldwell & Lyle |
| No difference | | Low | High | Plass & Mathew |

Slemons & Morriss observed that rises and falls in the maternal blood urea were always accompanied by like rises and falls in the foetal blood urea, and they concluded that urea passed into the maternal circulation by diffusion. As the above table shows, the concentrations of nitrogenous end products in the maternal and foetal blood are almost identical, with a slightly higher level in some cases in the foetal blood. If a gradient exists, then, it is in the direction foetus→mother. Kreidl & Mandl in 1904 summarised the reasons for believing that by far the greater part of the nitrogenous excretion of the foetus passes through the foetal kidneys. The interesting anatomical evidence for this, with its bearing on the function of the mesonephros, will be considered in the Section on placental permeability.

If, then, we cannot suppose that the nitrogenous end products which collect in the allantoic sac of the herbivorous mammal are the sum of all the nitrogenous end products of the embryo, there is not much use in relating them to its total weight, i.e. in attempting

to gain some notion of the relative intensity of its protein catabolism, as was so straightforward in the case of the chick. Nevertheless, the enterprise is not without interest, and Lindsay essayed it, using her own figures in conjunction with those of Paton, Watson & Kerr. The results are plotted in Fig. 341, where an unmistakably descending curve is seen, becoming asymptotic to the time axis. Exactly parallel to this are the data of Grünbaum, who worked with the cow, estimating the total nitrogen in the allantoic liquid at different stages of development. His figures, or rather the results calculated from them, are not readily incorporated in the graph of Fig. 341, but are given in Table 160.

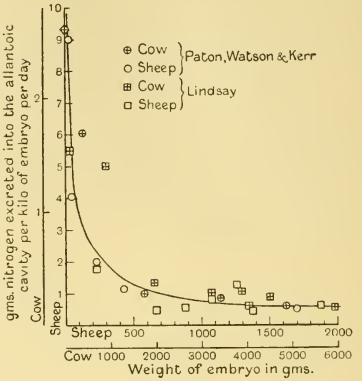



Fig. 341.

Table 160.

| Weight of the cow foetus in grams | Total nitrogen in the allantoic liquid in milligrams per 100 gm. of foetus weight |
|---|---|
| 0·9 | 9300 |
| 2·7 | 2070 |
| 4 | 1880 |
| 22 | 690 |
| 285 | 245 |
| 870 | 177 |
| 1,150 | 175 |
| 1,200 | 120 |
| 2,050 | 128 |
| 5,500 | 67 |
| 13,000 | 82 |

"The waste nitrogen", said Lindsay, "accumulates as the foetus grows, but per unit of weight it markedly decreases." This holds good for all the constituents of the urine, as found from the analysis of the allantoic and amniotic fluids, and a group of descending curves is seen in Fig. 342. Now, if the curve of Fig. 341 be compared with that given in Fig. 325 for the intensity of protein metabolism of the

chick embryo during its development, a striking resemblance appears, if it is assumed that the curve for the sheep and cow is the descending limb of a peaked curve essentially similar to that occurring in the chick embryo. If this view were adopted, we should have to conclude that the protein utilisation peak was reached by the sheep embryo some time before it attained the weight of 8 gm. (i.e. about the 4th week out of 22), and by the cow embryo before it attained the weight of 400 gm. (i.e. about the 12th week out of 32). In this there is nothing improbable. On the other hand, as Lindsay herself suggested, there is nothing to show that placental excretion does not play a relatively larger part towards the end of development, so that the fall in intensity of protein combustion might be purely an artifact. If this were the case, a difference in urea and uric acid content of the foetal blood in early and late pregnancy would be worth looking for. But, whether this be so or not, it is at any rate suggestive that we have a curve for the intensity of protein combustion for the mammalian embryo which looks as if it might be in perfect correspondence with that established for the oviparous avian embryo. It would be interesting to make calculations about the total quantity of waste nitrogen produced by the mammalian embryo during its intrauterine life, using the data for nitrogen content of amniotic and allantoic liquids, and for urea content of maternal and foetal blood, but such a calculation would be beset with so many difficulties, and would involve so many assumptions, that it is not worth beginning it in the present state of our knowledge. In view of the arguments to be brought forward at the end of this chapter, it might be predicted that the mammalian embryo would combust much more protein than that of the chick, but it would be difficult to know in what terms to express it, for the initial store could be regarded as almost unlimited, and the total material combusted would be extremely difficult to ascertain. Perhaps something could

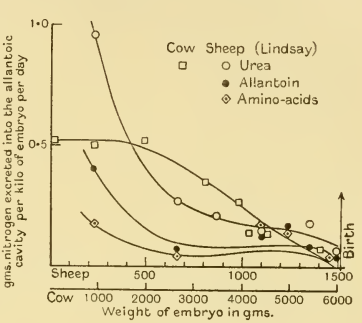



Fig. 342.

be done on the basis of a calculation giving the maximum amount of protein which the maternal apparatus could supply to the embryo in the time.

The data of Table 158, mentioned above, are in some ways rather difficult to interpret. The urea concentration in the amniotic liquid of the cow and sheep does not change much during pregnancy, nor does its percentage in terms of total nitrogen, though here there is a slight decrease. In the allantoic liquid the absolute quantity of urea is very much greater. The decrease in the proportion of urea nitrogen is more marked in the allantoic liquid of the cow than of the sheep. The amount of allantoin present in both liquids is considerable, and increases with length of pregnancy in the cow, but not in the sheep. The amino-acids, including hippuric acid, were abundant, especially towards the middle of pregnancy, in the allantoic liquids of sheep and cow, and they increased more rapidly the younger the foetus. In the cow the amino-acids make up the same proportion of the total nitrogen in the allantoic fluid, but rise in the amniotic, while in the sheep exactly the reverse process takes place. In the allantoic fluid of the sheep there was a considerable increase of hippuric acid as pregnancy advanced, both absolute and proportionate, but in the amniotic fluid the tendency was the other way.

"The picture of foetal metabolism", said Lindsay, "thus shown by the chemical composition of the early allantoic liquid is one of low deaminising power as indicated by the low urea output, the absence of ammonia, and the high proportion of amino-acids." A final remark which might be made before leaving these questions is that the very high allantoin content of the early allantoic urine might be taken to indicate an intense nucleoprotein metabolism (for here only purines would be concerned, unlike the bird), in which case the findings of LeBreton & Schaeffer (p. 1153) on the chemical nucleoplasmatic ratio would be seen in a new light.

Traces of proteins are found in the amniotic and allantoic liquids of mammals, but for this see Section 22.

### 9·14. Protein Utilisation of Explanted Embryonic Cells

The metabolism of mammalian embryonic cells in tissue culture has been studied by Holmes & Watchorn, in experiments which bear the same relation to the subject of this section as those of Cohn & Murray to the section on growth. They used kidney and

brain tissue from embryo rats, maintained *in vitro* with precautions ensuring sterility, and on these they carried out ammonia and urea estimations, making special provision for a complicated series of controls necessitated by the autolysis of the medium when held at 37°. Comparing the results from "resting" tissue, i.e. tissue submerged and floating so that it cannot grow but can yet live, with those from tissue attached to cotton-wool fibres and vigorously growing, Holmes & Watchorn found much more urea and ammonia production from the latter than from the former. Unfortunately, it was not usually practicable to weigh the pieces of kidney selected for culture, so that we cannot tell what level of metabolic intensity this represented, but in one instance 33 mgm. wet weight of kidney tissue had produced after a time unstated 0·023 mgm. of urea and 0·039 mgm. of ammonia, or about 0·05 mgm. of total nitrogen, i.e. 0·15 mgm. per 100 mgm. wet weight of the original tissue. Holmes & Watchorn found no evidence for the activity of urease in their cultures. In brain cultures, on the other hand, there was a definite suggestion of the action of urease, and when good growth took place there was found a surprising result, namely, that there was a considerable fall in the ammonia and urea nitrogen. Possibly a synthesis of nitrogenous substances such as choline was occurring. In a later paper these investigators added glucose to the medium of culture, and found that it gave rise to a marked alteration in the metabolism of the tissue, bringing about a definite inhibition of ammonia and urea formation. This was the second *in vitro* demonstration of the protein-sparing action of the carbohydrates: for the first see Warburg, Posener & Negelein (p. 765). Still later, Holmes & Watchorn used their technique to study the relation of cyanate, hydantoinacetic acid, etc., to urea and ammonia production by embryonic kidney cells *in vitro*.

As regards the nourishment of the mammalian egg-cell before its implantation into the uterine wall, Emrys-Roberts suggested that the secretion of the mammalian uterus was analogous to that of the avian oviduct, and supplied a kind of egg-white which the ovum could digest and dissolve. He made no experiments to prove the point, but he conceived that the gelatinous envelope which surrounds the embryo of the rabbit and the mole before implantation was produced by a fermentative and coagulating action on the part of the trophoblast. Sobotta and Caffier have produced evidence in favour of such a mechanism.

## 9·15. Uricotelic Metabolism and the Evolution of the Terrestrial Egg

We have now examined all the existing knowledge about the protein metabolism in embryonic life, and it is time to turn to a few general considerations. One of the questions always asked by students in biochemistry is why some animals should excrete urea, some ammonia, some uric acid. As far as I know, they have not so far been accustomed to receive any reasonable reply, and the problem has been set down as one of those arbitrary dispositions of fate which make Elementary Classes despair of biochemistry, but I think an answer can be given.

Fiske & Boyden, in their memoir on the nitrogen metabolism of the hen's egg, raised an interesting point when they calculated that 15 per cent. of all the water in the egg at the beginning is needed to excrete the 5 mgm. odd of uric acid which are present in the allantoic liquid by the 11th day of development. From that time onwards re-absorption of water vigorously proceeds, no doubt for the reason that, without it, all the water in the residues and in the body of the embryo would be required to get rid of the uric acid that is to be formed. It is as if the water acted as an endless belt conveyer, transferring uric acid from the cells of the embryo into the allantoic liquid, and then returning to transfer more. The fowl is always good at absorbing water from its excretions, for, as Wiener and Sharpe have shown, the glomerular urine in the adult is quite liquid, and the cloaca absorbs great quantities of water. All terrestrial animals do this to some extent, if the views of Cushny about the function of the mammalian kidney tubules are correct. But in the hen's egg it is obvious how closely the process as a whole is bound up with the properties of uric acid. "A substance as soluble and diffusible as urea", say Fiske & Boyden, "could not possibly replace it as an end-product when the organism and its excretions are confined to a closed system, the walls of which are only permeable to matter in the gaseous state."

This is a very important consideration. There appear to be only three substances which are available in the animal kingdom for carrying away the nitrogenous waste resulting from protein breakdown—ammonia, urea and uric acid. The first two of these compounds are very soluble and diffusible; uric acid is not. Quantitative expression of this fact has been given by Chauffard, Brodin &

Grigaut, who found a dialysis coefficient of 93 for urea, but only 74 for sodium urate. The haemato-encephalic barrier, according to them, allows urea to pass easily, but not uric acid. And the first substance retained in the mammalian circulation, if the kidneys are impaired, is uric acid. Shut up as it is in its closed box, the chick embryo would evidently find uric acid by far the most convenient excretory product, for the two former would tend to diffuse throughout the egg, and to establish themselves in equal concentration in all its constituent regions, instead of being packed into a small store. As it happens, the work of Kamei provides a striking verification of this viewpoint, for as we have seen, he showed that, in the amniotic liquid of the chick, although the uric acid concentration never rises above a certain very low level, the ammonia and the urea rise continuously throughout development. It is easy to guess, therefore, what would happen if all the nitrogen excreted by the embryo were in the form of urea. As an illustrative calculation we may take the uric acid present in the allantois at the end of incubation as 100 mgm. (data of Fiske & Boyden; Needham; Targonski and others)—i.e. about 33 mgm. of uric acid nitrogen or 66 mgm. of urea. This, distributed over an egg of contents approximately 40 gm., would be 165 mgm. per cent. The egg would be uraemic (in the strict, not the clinical, sense of the word). The normal figure for the urea-content of human and bovine blood is about 25 mgm. per cent., and the highest figure on record obtained by ingesting solid urea is just under 100 mgm. per cent. In severe renal obstruction or nephritis, it rises above 100, and may reach 300 or 400, but 165 is undoubtedly of the pathological order of magnitude, and if the avian embryo had to suffer from a constant headache and other symptoms before hatching, natural selection would hardly have preserved it for our entertainment. These consequences could be avoided by the use of uric acid.

Such considerations lead to the suggestion that the form of excretion of nitrogen adopted by an animal depends principally on the conditions under which its embryo has to live. There is good evidence that the combustion of protein substances as a source of energy is much more marked in aquatic than in terrestrial embryos (Needham). Table 161, constructed from as much of the information as is trustworthy, shows the partition between the substances comprising the total material catabolised. Thus only 5 or 6 per cent. of the total

matter combusted by the chick embryo is protein, but the frog embryo combusts as much as 71 per cent. during its embryonic life. Everything points to very deep-seated differences between eggs which develop in the water and eggs which develop on land. Not only do aquatic embryos burn much more protein in per cent. of the total material burned, but also in per cent. of the initial store of protein.

Table 161. *Material burned as source of energy in per cent. of the total material so burned.*

| | Carbohydrate | Protein | Fat | Investigators |
|---|---|---|---|---|
| Terrestrial. Chick (*Gallus domesticus*) | 3·02 | 5·57 | 91·4 | Murray; Needham; Fiske & Boyden |
| Aquatic. Frog (*Rana temporaria*) | 6·84 | 70·70 | 22·4 | Barthélemy & Bonnet; Fauré-Fremiet & Dragoiu; Bialascewicz & Mincóvna; Needham |
| Aquatic. Trout (*Savelinus fontinalis*) | — | 63 | 37 | Gortner |
| Aquatic. Plaice (*Pleuronectes platessa*) | — | 90 | — | Dakin & Dakin |
| Terrestrial. Silkworm (*Bombyx mori*) | — | 10 | 64 | Tichomirov; Farkas |
| Aquatic(?) Turtle (*Thalassochelys corticata*) | — | 19 | 81 | Tomita; Nakamura; Karashima |

The figures above the line are those most accurately known. Credit should be given to Halban as the first to suggest that there might be a fundamental difference between aquatic and terrestrial embryonic life.

Table 162 shows this very clearly. The embryos of the chick and the silkworm are the only terrestrial ones for which we have dependable figures, and they agree in burning about 4 per cent. of their initial store of protein. Among aquatic embryos, the frog, the trout and the plaice agree in burning about 25 per cent. In the case of embryos which hatch only half-way through their development, as most of the aquatic ones do, it is interesting to find that up to hatching their protein utilisation is not high, but that for the whole embryonic period it much exceeds that of terrestrial embryos. Thus there is reason for supposing that the terrestrial environment of the embryo has two effects on its protein metabolism; firstly, to suppress the production of nitrogenous waste by removing the means of its easy disposal, and, secondly, to elevate uric acid to the place of importance as a means of excreting nitrogen. From this point of view, the invention of viviparity was a "back-to-the-sea" movement on

the part of the embryo, for even if, as McCallum would have us believe, the maternal sea water is practically pre-Cambrian, it is at any rate as good as any other sea water for the disposal of nitrogenous waste products, and from the embryonic point of view, a boundless ocean. In other words, the continuous perfusion system of the vivipara provides an artificial sea, and avoids the necessity of a

Table 162. *Protein burned in per cent. of the initial protein store.*

| Animal | Aquatic or terrestrial embryo | Protein nitrogen combusted during development in % of the total protein nitrogen present at the beginning | | Investigator |
|---|---|---|---|---|
| PISCES. Brook-trout (*Salvelinus fontinalis*) | A. | To hatching | 3·4 | Gortner |
| | | To end of yolk-sac period | 21·9 | ,, |
| | | To end of yolk-sac period | 17·0 | Pearse |
| PISCES. Plaice (*Pleuronectes platessa*) | A. | To end of development | 18·3 | Dakin & Dakin |
| AMPHIBIA. Frog (*Rana temporaria*) | A. | To disappearance of external gills | 25·7 | Barthélemy & Bonnet |
| | A. | To hatching | 9·1 | Bialascewicz & Mincóvna |
| | | To end of yolk-sac period | 40·0 | |
| | A. | To hatching | 10.6 | Fauré-Fremiet & Dragoiu |
| | | To end of yolk-sac period | 23·1 | |
| | A. | To hatching | 9·2 | Fauré-Fremiet & du Streel |
| AMPHIBIA. Salamander (*Cryptobranchus allegheniensis*) | A. | To hatching | 4·9 | Gortner |
| INSECTA. Silkworm (*Bombyx mori*) | T. | Whole development | 3·9 | Russo |
| CHELONIA. Turtle (*Thalassochelys corticata*) | prob. A. | (By end products found and nitrogen lost) | 16·5 | Tomita; Nakamura |
| AVES. Chick (*Gallus domesticus*) | T. | (Indirect calculations) | 5·8 | Idzumi |
| | T. | (By protein lost) | 8·0 | Sakuragi |
| (All figures are for the whole of development) | T. | (By end products found) | 1·1 | Needham |
| | T. | (By end products found) | 3·4 | Fiske & Boyden |

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uricotelic metabolism. Is it surprising, in view of these facts, that fishes turn the ammonia from their protein breakdown into urea, birds and reptiles into uric acid, and mammals once more into urea?

The thought may be stated in another way. Perhaps the sauropsida excrete their nitrogen mainly as uric acid because they had to learn how to do so in order to pack their embryos into solid- and

liquid-tight boxes, and never afterwards forgot. Even the eggs of water birds, which might be supposed to have the opportunity of excreting substances into the water around them, have impenetrable fat-impregnated shells, as Loisel has shown. The highest avian groups, exemplifying as they do the most complicated form of nitrogen excretion, would thus represent the crowning achievements of the uricotelic line of evolution. And the fact that, between the 2nd and 5th days in the chick's development, it excretes ammonia and urea with no uric acid would thus be a recapitulation of its pre-terrestrial or aquatic ancestry, entirely analogous with its gill-clefts. Moreover, the coincidence is exact, for it is just between the 2nd and 5th days that the embryo manifests its morphologically piscine characteristics. Fig. 323 showed the milligrams of ammonia, urea and uric acid present in embryo, amniotic and allantoic liquid throughout incubation, expressed in terms of 100 gm. dry weight of embryo; in other words, it showed what 100 gm. dry weight of embryo has manufactured in the way of nitrogenous end products by any given time. Table 138 showed the relations between these substances in another way. Although ammonia, urea and uric acid are excreted by the chick embryo during its development, the two first-named molecules only account for an insignificant part of the total nitrogen excreted. There is a progression in ontogeny from the smallest to the largest molecule, and from the most to the least efficient excretory product. It could be argued, of course, that, if the chick can excrete urea early in its development, it ought to be able to return to this practice after hatching; but this would be to neglect one of the most characteristic features of embryonic life, namely, its continual tendency to lose pluripotence, and to move towards a stable, "crystalline", or set state. The chick embryo begins to excrete uric acid about the 6th day. Before that time, as we know from the work of Przylecki & Rogalski, it possesses uricase, but after that time it does not. Perhaps, then, when the reptiles came ashore they found it was as well to leave their uricase behind them. As for avian development, hatching is followed by a sudden burst of protein catabolism, as we have seen in Section 6·11, and this is probably associated with the chick's enlarged opportunities for getting rid of nitrogenous waste.

There remains the consideration that no animal would excrete uric acid as its main nitrogenous end product unless it was driven to it,

for of the three in actual use it is much the most wasteful. Ammonia is clearly the most efficient end product, for it involves no wastage of carbon, but of the other two the carbon/nitrogen ratio is 1/2 in urea and 1/0·9 in uric acid. In other words, two atoms of nitrogen can be got rid of at the expense of only one carbon atom in urea, but only 0·9 in uric acid. These amounts may not be individually considerable, but collectively they may make all the difference between an efficient and an inefficient species. Ackermann has compiled an interesting table showing the nitrogen-removing efficiencies of many urinary constituents, and has emphasised this point. Moreover, it is obvious that uric acid excretion involves the wastage of a great deal more chemical energy than urea, thus:

| | Heat of combustion (cals. per gm. mol.) |
|---|---|
| Ammonia | 90·6 |
| Urea | 152·6 |
| Uric acid | 462·1 |

Uric acid, then, as the main end product of protein metabolism, may be said to be more ingenious than the other two, but less efficient.

The proposition that the circumstances in which the embryonic life has to be passed ultimately govern the form in which the nitrogen is excreted is thus not so far-fetched as it sounds. During the last fifty years much attention has been paid to the comparative study of nitrogen excretion, but the methods of the older workers, such as Krukenberg and Griffiths, were so unreliable that the earlier literature may be neglected. More recently, the researches of Przylecki; Delaunay and others have begun the erection of a solid structure of knowledge about the forms in which nitrogen is excreted. We are thus acquiring, as it were, a wide series of phylogenetic base-lines on which ontogenetic phenomena can be superimposed. The general conclusions of these workers support the idea of an association between aquatic life and the excretion of ammonia and urea, on the one hand, and between terrestrial life and the excretion of uric acid, on the other hand. But the important point is that the life of the embryo is the key, not the life of the adult. An animal may live all its life in the sea, but if its eggs are laid and develop on land it may be predicted that its main nitrogenous end product will be uric acid. Mammals, from the chemico-embryological viewpoint, count as aquatic animals, since the excretion of nitrogenous waste products through the placenta is analogous to their excretion into water.

Another interesting fact which fits in closely with this point of view is that, according to the researches of Przylecki who investigated a large variety of organisms, no animal possesses both uricoligase and uricase. In other words, all those animals which possess the power of making uric acid from amino-acids cannot destroy it, and all those which can destroy it have no power of making it, other than from purines. This certainly looks as if the power of formation of uric acid from amino-acids was an adaptation of evolutionary value, for if uricase and uricoligase were often present together, it would be difficult to suggest that any special advantage was to be gained in certain circumstances from the manufacture of uric acid.

In Table 163 are collected together a number of figures for nitrogen excretion in various animals. All the older work has been excluded, and, as far as possible, only quantitative investigations of the percentage distribution of the excretory nitrogen appear. As a general rule, the marine invertebrates excrete most of their nitrogen as ammonia—a simple and easy procedure, considering their environment. But with the increasing complexity of the body, ammonia excretion disappears, for it is incompatible with a kidney, even in a very undeveloped form. Excretory structures—structures which have to live, as it were, in an excretory atmosphere—cannot deal with highly alkaline liquids, and the great disadvantage about simple ammonia excretion is that a constant supply of acid is required to neutralise it. This acid is nothing but waste, and so among the marine invertebrates themselves we see urea superseding ammonia. Among the invertebrates the only ones at present known which have a high percentage of uric acid are the pulmonate gastropods, the snail and the slug, which live on land and have terrestrial embryos. In this connection the concretions of urates in certain snails, which, according to McKinnon, contain bacteria capable of breaking down uric acid, are of special interest. Delaunay himself pointed out that the invertebrates could be separated into an aquatic and a terrestrial group, the former excreting much ammonia and the latter little, and he also remarked on the association between uric acid and terrestrial life. But this might remain enigmatic if we did not consider the needs of the *embryo*; able, in the one case, to get rid of its nitrogenous waste easily into the surrounding water, and forced, in the other case, to keep it close at hand in very restricted quarters. Delaunay's generalisation alone would not explain the

Table 163. *Quantitative Data for Nitrogen Partition in Urine.*

| Animal | Aquatic or Terrestrial eggs | Nitrogen Partition in % of the total nitrogen excreted: Ammonia | Urea | Uric acid | Amino-acids, creatine & creatinine | Purines other than uric acid | Investigator |
|---|---|---|---|---|---|---|---|
| PROTOZOA | | | | | | | |
| *Paramoecium* ... ... ... | — | — | 90·0 | None | — | — | Weatherby |
| *Didinium* ... ... ... | — | 90·0 | — | None | — | — | ,, |
| ANNELIDA | | | | | | | |
| Sea-mouse (*Aphrodite aculeata*) | A. | 80·0 | 0·2 | 0·8 | — | — | Delaunay |
| Leech (*Hirudo officinalis*) ... | A. | 76·4 | 5·4 | None | 3·2 | 3·6 | ,, |
| Earthworm (*Lumbricus agricola*) | ? | 20·4 | 38·1 | Trace | 15·8 | 9·3 | ,, |
| GEPHYREA | | | | | | | |
| Worm (*Sipunculus nudus*) ... | A. | 50·0 | 9·7 | None | 16·6 | 4·1 | ,, |
| ARTHROPODA | | | | | | | |
| Crustacea | | | | | | | |
| Crab (*Carcinus moenas*) ... | A. | 67·8 | 2·9 | 0·7 | 8·7 | 2·3 | ,, |
| Spidercrab (*Maia squinado*)... | A. | 42·9 | 5·2 | 2·7 | 20·2 | 3·5 | ,, |
| Crayfish (*Astacus fluviatilis*) ... | A. | 59·6 | 11·2 | 0·8 | 10·1 | 3·7 | ,, |
| Insecta | | | | | | | |
| Silkworm (*Bombyx mori*) ... | T. | — | None | 85·8 | — | — | Farkas |
| Clothes-moth (*Tinea pellionella*) | T. | 10·22 | 17·6 | 47·3 | 10·0 | 0·51 | Babcock & McCollum |
| Clothes-moth (*Tinella crinella*) | T. | 20·7 | 1·8 | 77·5 | — | — | Hollande & Cordebard |
| Insects in general ... ... | T. | "Als characterisches Stoffwechselendprodukt des Insektenorganismus ist die Harnsäure zu betrachten." | | | | | von Fürth* |
| MOLLUSCA | | | | | | | |
| Gastropoda | | | | | | | |
| Sea-hare (*Aplysia limacina*) ... | A. | 33·5 | 8·7 | 4·6 | 13·0 | 9·3 | Delaunay |
| Land snail (*Helix pomatia*) ... | T. | 13·7 | 20·0 | 10·7 | 6·0 | 5·8 | ,, |
| Slug (*Limax agrestis*) ... | T. | 4·6 | 70·8 | 6·9 | 1·7 | 5·9 | ,, |
| Lamellibranchiata | | | | | | | |
| Clam (*Mya arenaria*) ... | A. | 21·5 | 4·5 | Trace | 18·0 | 5·0 | ,, |
| Oyster (*Gryphoea angulata*) ... | A. | 7·3 | 3·2 | 0·2 | — | — | ,, |
| Pond-mussel (*Anodonta cygnaea*) | A. | 63·0 | — | — | — | — | Przylecki |
| Cephalopoda | | | | | | | |
| Octopus (*Octopus vulgaris*) ... | A. | 33·3 | — | 1·4 | 12·5 | 23·6 | Delaunay |
| ,, ,, | | 41·7 | 15·0 | — | 20·7 | — | von Fürth |
| Octopus (*Sepia officinalis*) ... | A. | 67·0 | 1·7 | 2·1 | 7·8 | 1·9 | Delaunay |
| ECHINODERMATA | | | | | | | |
| Starfish (*Asterias rubens*) ... | A. | 39·3 | 11·7 | Trace | 23·8 | 6·8 | ,, |
| Sea-urchin (*Paracentrotus lividus*) ... | A. | 28·1 | 7·5 | 1·0 | 28·0 | 10·0 | ,, |
| Sea-cucumber (*Holothuria tubulosa*) ... | A. | 40·0 | 6·0 | Trace | — | — | ,, |

* p. 295. See also Ackermann; Kawase & Suda; Kutscher & Ackermann; Roubaud; Poisson; and Schmieder.

Table 163. *Quantitative Data for Nitrogen Partition in Urine* (cont.).

| Animal | Aquatic or Terrestrial egg | Nitrogen Partition in % of the total nitrogen excreted: Ammonia | Urea | Uric acid | Amino-acids, creatine & creatinine | Purines other than uric acid | Investigator |
|---|---|---|---|---|---|---|---|
| VERTEBRATA | | | | | | | |
| Pisces | | | | | | | |
| Pipefish (*Muraena helena*) ... | A. | 23·7 | 30·0 | 0·0 | 28·0 & 15·0 undetermined | | Edwards & Condorelli |
| Goosefish (*Lophius piscatorius*) | A. | 36·8 | 16·2 | 0·0 | 15·8 & 29·2 undetermined | | ,, ,, |
| ,, ,, | A. | 0·5 | 0·7 | 0·4 | 46·3 & 52·1 of trimethylamine oxide* | | Grollmann |
| ,, ,, | A. | — | — | — | 23–56 and a great deal of undetermined | | Marshall & Grafflin |
| ,, ,, | A. | 13·0 | 62·0 | 0·1 | — | — | Denis |
| Dogfish (*Mustelus canis*) ... | A. | 7·3 | 80·7 | 0·2 | — | — | ,, |
| Dogfish (*Scyllium canicula*) ... | A. | — | 80·0 | 0·0 | — | — | Herter |
| Carp (*Cyprinus carpio*)† ... | A. | 56·0 | 5·7 | 0·2 | 10·6 & 28·2 undetermined | | Smith |
| ,, ,, ... | A. | 77·4 | 14·5 | — | 2·6 | — | Delaunay |
| Sole (*Solea vulgaris*) ... ... | A. | 53·0 | 16·6 | — | — | — | ,, |
| Seahorse (*Hippocampus*) ... | A. | 63·1 | 8·9 | — | 13·8 | — | ,, |
| Angler-fish ... ... ... | A. | 2·0 | 28·0 | — | 5·0 | — | ,, |
| Torpedo (*Torpedo*) ... ... | A. | 3·8 | 91·4 | — | 0·8 | — | ,, |
| Lung-fish (*Protopterus aethiopicus*) ... ... ... | A. | 41·2 | 18·5 | 0·8 | 7·0 | | Smith |
| Amphibia | | | | | | | |
| Frog (*Rana temporaria*) ... | A. | 15·0 | 82·0 | Trace | — | — | Przylecki |
| ,, ,, | A. | 5·1 | 87·5 | 0·2 | 7·25 undetermined | | Toda & Taguchi |
| Frog (*Rana virescens*) ... | A. | 3·2 | 84·0 | 0·4 | — | — | van der Heyde |
| Toad (*Bufo vulgaris*) ... | A. | — | 84·5 | Trace | — | — | Burian |
| Reptilia | | | | | | | |
| Chelonia | | | | | | | |
| Turtle (*Chrysemys pinta*) ... | A. | 15·3 | 39·0 | 18·8 | 11·5 & 18·6 undetermined | | Wiley & Lewis |
| Alligator (*Alligator mississipiensis*) ... ... ... | A. | 75·3 | 7·2 | 13·1 | — | — | Hopping |
| Turtle (*Chelone mydas*) ... | A. | 16·1 | 45·1 | 19·1 | — | — | Lewis |
| Tortoise (*Testudo graeca*) ... | A. | — | 90·0 | Trace | — | — | Clementi |
| Sauria | | | | | | | |
| Snake (*Boa constrictor*) ... | T. | — | — | 80·0 | — | — | Boussingault |
| Snake (*Python*) ... ... | T. | — | — | 80·0 | — | — | ,, |
| Snake (*Python*) ... | T. | 8·7 | — | 89·0‡ | 2·3 | — | Bacon |
| Grass-snake (*Tropidonotus natrix*) ... ... ... | T. | Trace | Trace | 80·0 | — | — | Girod |

* It has been suggested that this extraordinary nitrogen partition may be an adaptation; trimethylamine as an odorous substance being used to attract the prey. Grollmann's figures apply only to the product of the kidneys and do not include that of the gills.

† Fishes were found by H. W. Smith to excrete 6–10 times as much nitrogen through the gills as through the kidneys: the former dealt with the readily diffusible end products and the latter with the rest. According to Delaunay, Selachians excrete mostly urea with little ammonia and teleosteans mostly ammonia with little urea.

‡ In these cases the ammonia present was calculated to be just sufficient to combine with the uric acid as ammonium urate.

Table 163. *Quantitative Data for Nitrogen Partition in Urine* (cont.).

| Animal | Aquatic or Terrestrial egg | Nitrogen Partition in % of the total nitrogen excreted: Ammonia | Urea | Uric acid | Amino-acids, creatine & creatinine | Purines other than uric acid | Investigator |
|---|---|---|---|---|---|---|---|
| VERTEBRATA (*cont.*) | | | | | | | |
| Reptilia (*cont.*) | | | | | | | |
| Sauria (*cont.*) | | | | | | | |
| Lizard (*Lacerta viridis*) ... | T. | — | — | 91·0 | — | — | von Schreiber |
| Horned lizard (*Phrynosoma cornutum*) ... | T. | 20·0 | None | 98·0* | — | — | Weese |
| Aves | | | | | | | |
| Hen (*Gallus domesticus*) ... | T. | 1·5 | 0·9 | 70·0 | — | — | Salaskin & Kovalevski |
| ,, ,, | — | — | 1·1 | 62·9 | — | — | Minkovski |
| ,, ,, | — | 17·3 | 10·4 | 62·9 | 9·4 | — | Davis |
| ,, ,, | — | — | — | 65·7 | 6·0 | — | Mayrs |
| Swan (*Cygnus*) ... ... | T. | 15·8 | 2·6 | 68·7 | — | — | Salaskin & Kovalevski |
| Duck (*Anas*) ... ... | T. | 3·2 | 4·2 | 71·9 | — | — | Szalagyi & Kriwuscha |
| Goose (*Anser*) ... ... | T. | 13·5 | — | 80·0 | — | — | Paton |
| Mammalia | | | | | | | |
| Man (*Homo sapiens*) ... | A. | 4·3 | 87·5 | 0·8 | — | — | Folin |
| Dog (*Canis vulgaris*) ... | A. | 3·0 | 89·0 | 1·0† | — | — | Osterberg & Wolf |
| Cat (*Felis vulgaris*) ... ... | A. | 4·9 | 68·1 | 0·1 | — | — | Hammett |
| Badger (*Toxidia texus*) ... | A. | — | — | 8·0 | — | — | Hunter, Givens & Guion |
| Raccoon (*Procyon lotor*) ... | A. | — | — | 3·0 | — | — | ,, ,, |
| Guinea-pig (*Cavia*) ... ... | A. | — | — | 3·5 | — | — | ,, ,, |
| Rat (*Mus rattus*) ... ... | A. | — | — | 5·0 | — | — | ,, ,, |
| Opossum (*Didelphys virginiana*) ... ... ... | A. | — | — | 1·5 | — | — | ,, ,, |
| Horse (*Equus caballus*) ... | A. | — | — | 3·2 | — | — | ,, ,, |
| Sheep (*Ovis vulgaris*) ... | A. | — | — | 3·3 | — | — | ,, ,, |
| Whale (*Balaena mysticetus*) ... | A. | 1·5 | 90·0 | 3·0 | — | — | Schmidt-Nielsen & Holmsen |
| ,, ,, | A. | 3·5 | 91·0 | 0·3 | 1·5 | — | Ichimi, etc. |
| Egyptian bat (*Xantharpyia collaris*) ... ... ... | A. | 0·6 | 77·8 | 1·2 | | — | Popp |
| Dromedary (*Camelus dromedarius*) ... ... ... | A. | 12·3 | 55·5 | 0·3 | 19·3 | — | Smith & Silvette |
| Camel (*Camelus bactrianus*) ... | A. | 0·5 | 97·1 | 2·4 | — | — | Petri |
| | A. | 4·1 | 62·7 | 1·0 | 18·5 | — | Smith & Silvette |
| Weasel (*Putorius vulgaris*) ... | A. | 0·9 | 85·2 | 0·2 | 2·1 | 0·6 | Fuse |
| Tiger (*Felis tigris*) ... ... | A. | 3·7 | 89·1 | 0·04 | 1·9 | 0·2 | ,, |
| Hyaena (*Hyaena crocuta*) ... | A. | 3·8 | 89·3 | 0·1 | 1·5 | 0·2 | ,, |
| Llama (*Auchenia huanacos*) ... | A. | 2·2 | 67·7 | 0·8 | 10·0 | — | Smith & Silvette |
| Alpaca (*Auchenia vicunna*) ... | A. | 4·5 | 59·6 | 0·3 | 8·0 | — | ,, ,, |
| Monotreme | | | | | | | |
| Spiny anteater (*Echidna aculeata*) ... ... | ? | 6·9 | 81·2 | None | — | — | Neumeister |
| ,, ,, | ? | — | 88·0 | Some | — | — | Robertson |

* In these cases the ammonia present was calculated to be just sufficient to combine with the uric acid as ammonium urate.

† In mammals other than the primates the purine ring is excreted in the form of allantoin but this is not shown in the Table.

mammals. It is the conditions under which the *embryo* has to live that govern what form of nitrogen shall be excreted throughout the life-span. As Table 163 shows, the fishes and mammals (even the whale) with their urea and the birds with their uric acid fit in with the theory here propounded. The insects also are in perfect accord, for, although we have no quantitative data concerning the nitrogen partition of their urine, yet it has been generally recognised for many years that uric acid is the most prominent constituent of their excreta (see von Fürth), and there is doubt if urea has ever even been shown to be present. A quite parallel case is that of the hymenoptera and diptera, which excrete uric acid during metamorphosis into their fat bodies, according to Fabre; Schmieder and other entomologists. Thus the insects, coming to live on land earlier than the reptiles, had the same chemico-embryological problem to face, and solved it in the same way. This is a good biochemical instance of the phenomenon known to zoologists as "convergence". It is interesting that ovoviviparity occurs among the insects, but never true viviparity (Holmgren; Keilin), and one would like to know why they never invented the placenta, and dropped their uricotelic qualities, for they did invent the "private pond", or amnion. The reptiles themselves form an interesting group in Table 163, for the chelonia, standing as they do phylogenetically near the amphibia, show a high ammonia and urea excretion, while the sauria with their terrestrial embryos show a high uric acid excretion. It is, of course, among the reptilian group that such a difference would be likely to show itself, as they were the first vertebrates to conquer the land. In this connection it may be recalled that Tomita found urea in the imperfectly cleidoic egg of a marine turtle. Clementi, again, has found arginase in chelonian livers, an enzyme which does not occur in the livers of uricotelic animals.

The eggs of aquatic animals seem to divide into two classes. The frog and the plaice develop within membranes which readily allow the nitrogenous end products to escape; the trout and *Ascaris* (Kozmina) do not. But hatching always occurs long before the end of development in these aquatic forms, so that the permeability of the egg-membranes is unimportant from the point of view of nitrogen excretion. The trout at hatching gets rid of what has accumulated, and for the rest of its embryonic life can excrete directly into the water. The elasmobranchs, which excrete their urea into their yolk

(Needham & Needham), would form a third class, but they are notable exceptions in many ways, and to describe their behaviour in terms of the language here used would be to say that they use their own blood and yolk as the sea, and pile up the end products of their protein metabolism within themselves.

The amphibia never broke loose from the fishes; they always retained a piscine larval stage and laid their eggs in water. But when the first reptiles[1] left the sea, they were faced with one or two very difficult embryological problems. To begin with they had to find out how to abandon metamorphosis, and to discover a way of arranging a water supply for their embryos. As Gray has shown, aquatic embryos depend upon their environment for a supply of water; in other words, the fertilised egg contains enough solid, but not enough water, to make the finished larva. The first terrestrial eggs, therefore, had to contain enough water as well as enough solid, and, as arrangements to prevent undue evaporation were essential, the closed-box system inevitably developed. The mechanism by which a constant pressure-head of water was provided in the terrestrial egg, namely, the egg-white, can be seen functioning at the present time in the yet unidentified acid, which, introduced by the embryo's metabolism into the egg-white, as Vladimirov has shown, gradually brings the latter to its isoelectric point, and liberates water by degrees from the colloidal albumen. All the economy of the successful terrestrial egg had to be directed towards conserving the water, and while a great bath would have been required to keep the urea concentration down within bearable limits, if all the nitrogen was excreted in that form, only 20 per cent. of the water in the egg need be set aside for handling uric acid. Another way out of the difficulty would have been to burn no protein at all, and therefore to avoid all incombustible residues, and it is possible that some of the extinct saurians explored the possibilities in this direction, but I suspect that some factor which as yet we cannot quite define dictates from within the cells that life without protein combustion is not possible. It is therefore likely that such reptilian experiments did not proceed very far. In this way the closed-box system with its partial suppression of protein metabolism and its uricotelic qualities came into being

No doubt the first terrestrial vertebrates laid their eggs in water, and probably, like the trout, they hatched early. But although this

[1] Or rather, their stegocephalic predecessors.

system may suffice in the sea, an embryonic reptile with a bag of yolk almost as big as itself would have, owing to obvious difficulties of locomotion, very little chance of survival on land. Such imperfectly mobile eggs would have been too tempting for adults of other species. The terrestrial egg had therefore to be constructed in such a way that the young organism could stay inside a long time and hatch out substantially mature. It had no chance, therefore, either to get rid of nitrogenous excreta at an early stage by hatching, or to excrete them through a semipermeable membrane. The only solution of the problem was uric acid. When, at a still later date, the prototheria and metatheria branched off from the reptiles in the mammalian direction, acquiring at last true viviparity, the need for a uricotelic metabolism ceased. For it is to be noted that there is no reason why an adult land animal should not excrete urea, if it drinks sufficient water, or even ammonia, if it has enough acid to spare. Thus Ambard found that a cat or dog on a meat diet, if left to itself, drinks exactly enough water to excrete urea at its maximum normal concentration, i.e. just to avoid the slightest uraemia. This is Ambard's "volume obligatoire". But what the adult does will depend on what it had to do as an embryo; in other words, on what its facilities then were for absorbing water and for getting rid of waste nitrogen. The existence of an albuminous solution round the yolk of the terrestrial egg is, as Gray says, an admirably adapted mechanism for providing the growing embryo with water. The use of uric acid—insoluble, non-diffusible—instead of urea or ammonia, is an equally well adapted mechanism for dealing with incombustible waste, and the re-absorption of water through the allantoic wall is the mechanism which unites the two. There is no need, however, to postulate any reversion from uricotelic to ureotelic metabolism in the case of mammals, for the palaeontological evidence admits of the possibility that they arose from some early reptile[1] which laid non-cleidoic eggs. True viviparity may thus have been a genuine alternative to uric acid production.

A final reference may be made to certain special cases, e.g. the Prototheria and the Elasmobranchs. The Elasmobranchs are at one and the same time the only marine animals which have evolved the closed-box system or something approaching it and the only ones which have found out a way to withstand high concentrations of urea. There are, perhaps, two possible explanations of their behaviour.

[1] Probably one or more lines of cynodont (Therapsid) reptiles.

They may first have discovered how to become permanently uraemic without suffering from it, and then have utilised the associated advantage of protecting their embryos until a late stage of development. Or they may have adopted a protective closed box, and then become adapted in some way to withstand the consequent uraemia. In any case, they offer an interesting comment on the terrestrial egg, for they seem to have found out a way of avoiding the uricotelic qualities of the closed-box system—a way, however, which appears to have been only suitable for a very restricted class of animals.[1]

As for the Prototheria, their excretory nitrogen as Table 163 shows, works out at a typically mammalian partition. As the *Echidna* is, strictly speaking, oviparous, this would seem at first sight to be much in opposition to the general views here suggested, but closer examination shows that this is not so. The *Echidna* lays eggs, it is true, but, according to Caldwell, who gives all the literature in his well-known paper, it picks them up and immediately places them in its pouch. Once there, they emerge at a very early stage from the thin shells, and suck up the milk which exudes from scattered pores inside the pouch. There seems no reason why the shells themselves should not be permeable to excretory products (for they are not hard), and after hatching there would not even be this difficulty—in both cases the epithelium of the pouch would be able to absorb the foetal excreta. The pouch, in fact, may be regarded as a uterus located in an unusual position, and, from the standpoint of this discussion, *Echidna* would be viviparous, and therefore "aquatic". It would be very interesting to investigate the nitrogen partition in the urine of *Ornithorhyncus* which allows its eggs to develop outside the body (Wood-Jones).

A generalisation might then be provisionally enunciated as follows: The main nitrogenous excretory product of an animal depends on the conditions under which its embryos live, ammonia and urea being associated with aquatic pre-natal life, and uric acid being associated with terrestrial pre-natal life. Up to the present time there has been no trace of order or system among the facts obtained by comparative investigations on nitrogen excretion, and no answer to the question of why a uricotelic metabolism should exist at all. The answer here suggested is that terrestrial oviparous animals would have been impossible without it.

[1] In this class the Dipnoi may perhaps be included. Smith has shown that the lungfish *Protopterus*, aestivating in its burrow, accumulates relatively enormous amounts of urea in its tissues.

SECTION 10

# THE METABOLISM OF NUCLEIN AND THE NITROGENOUS EXTRACTIVES

## 10·1. Nuclein Metabolism of the Chick Embryo

It has already been recounted in Section 1 (see p. 336) that many investigators, following Kossel, have not been able to isolate any purine bases from the unincubated hen's egg. At the present time it is generally agreed that not more than 2 per cent. of the total nitrogen at the beginning of development is in the form of purine bases. All investigators have agreed, moreover, that by the end of development purine bases are present in the embryo in considerable quantity, and there is no doubt that, during the embryonic growth of the chick, purine bases are synthesised. The only dissentient voice which breaks this unanimity is that of Mesernitzki, who maintained in 1903 that as much purine (estimated as xanthine) was present in the egg at the beginning as in the embryo at the end, but we may disregard it, for Fridericia adversely criticised his technique, and repeated his experiment with contrary results.

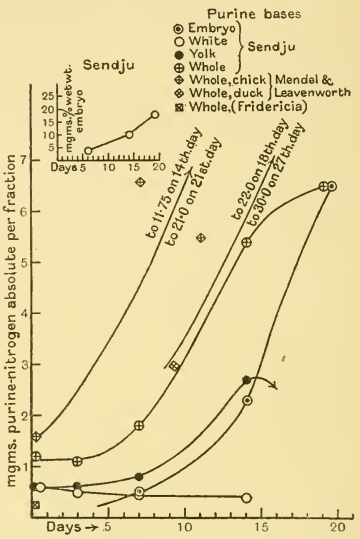


Fig. 343.

Sendju's paper contains figures for the purine nitrogen in white, yolk and embryo, and is probably the best one with which to introduce the subject. As is evident from Fig. 343, there is never more than a minimal quantity of the bases in the egg-white, but in the yolk, on the contrary, there is a good deal, and on the 14th day it

contains as much as the embryo. The graph accordingly resembles that for glycogen, for it is of the type in which the total amount of the substance increases, while the amount of the substance outside the embryo first increases and then decreases. Probably the reason why the yolk has so much is that the yolk-sac and its vessels were included in the analysis, and consequently the purine bases from the nucleoprotein of their cells were included. Mendel & Leavenworth have also estimated the purines in the avian egg, and their figures, which appear in Fig. 343, agree with Sendju's as demonstrating a vigorous synthesis, although in absolute value they much exceed his, by 22·0 to 7·0 on the last day of development. This is due to the fact that Mendel & Leavenworth probably included the uric acid present.

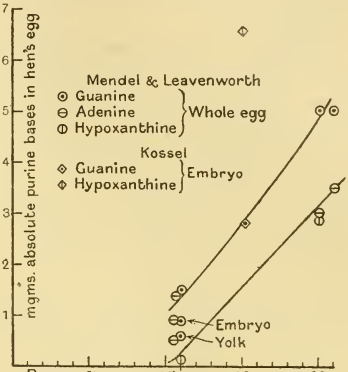



Fig. 344.

Mendel & Leavenworth separated a number of the individual bases by the Kruger-Schmid process, with the results shown in Fig. 344. Kossel's original figures are placed beside them for comparison. The concentration of the bases in the embryonic tissue was, it seemed, about the same as in adult tissue, thus:

| | Grams % wet weight | | | | |
|---|---|---|---|---|---|
| | Guanine | Adenine | Hypo-xanthine | Xanthine | |
| Cow muscle: embryo ... | 0·044 | — | 0·038 | 0·012 | Kossel, 1884 |
| adult ... ... | 0·005 | — | 0·053 | 0·012 | ,, |
| Pig muscle: embryo (100 mm.) | 0·093 | 0·065 | 0·029 | — | Mendel & |
| adult ... ... | 0·027 | — | 0·059 | — | Leavenworth, 1908 |

Wells & Corper remarked in 1909 that foetal tissues seemed to contain much more guanine than adenine, since on autolysis they gave twice as much xanthine as hypoxanthine. No more recent investigations have been made exactly along these lines, but Calvery has reported the presence of pentose nucleic acid with its pyrimidine,

uracil (the so-called "yeast nucleic acid"), in the 18th-day chick embryo, as well as the ordinary hexose nucleic acid similar to that first isolated from the thymus. The chick embryo thus possesses the power of synthesising both kinds of nucleic acid. The older observations of Mendel & Leavenworth therefore regain their importance, for, using Tollens' method, they found an increase in the pentose content of the incubated egg from zero to as much as 40 mgm. (see Fig. 345).

The first systematic investigation of the synthesis of nuclein bases by the embryo was made by Fridericia, who estimated them in the chick embryo from the 8th day onwards, using the Brugsch-Schittenhelm technique. He fully confirmed the earlier statements that not more than traces of purine bases were present in the unincubated egg, e.g. 0·25 mgm. purine nitrogen per egg. In order to determine whether the presence of any purine bases tightly enough combined to resist acid hydrolysis had been overlooked, Fridericia allowed fresh eggs to autolyse, but did not obtain in this way any more purine nitrogen than by the previous method. The small amount of nucleoprotein in the fresh egg is doubtless to be accounted for by the blastoderm itself. His figures for increase of purine nitrogen in the embryo are given in Fig. 346. Fridericia compared together in one and the same graph the absolute amounts of dry weight, total nitrogen and purine nitrogen. From the 8th to the 11th days the rate of increase was much the same, but after that the dry weight and total nitrogen curves drew away, and the purine nitrogen one was left slowly increasing below them. This obviously indicated that the purine nitrogen would decrease markedly in per cent. of the total nitrogen, but Fridericia did not pursue the subject further. Its importance will shortly appear.

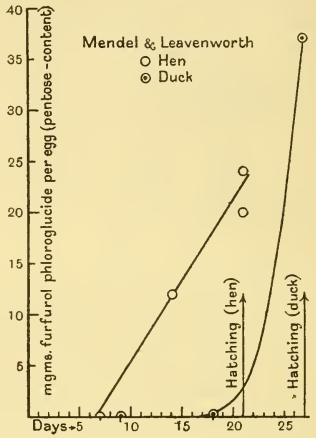


Fig. 345.

More recently, the question of nucleoprotein formation has been

examined by LeBreton & Schaeffer, who made a critical study of the possible methods, and eventually used their own modifications of the Kruger-Schittenhelm method. Their programme involved the estimation of the total nitrogen and the purine nitrogen at different stages in several series of embryos. Their data on the total nitrogen of the chick have already been given in Table 136 of Section 9·3, and their findings with regard to its increase in purine bases are shown in Fig. 346.

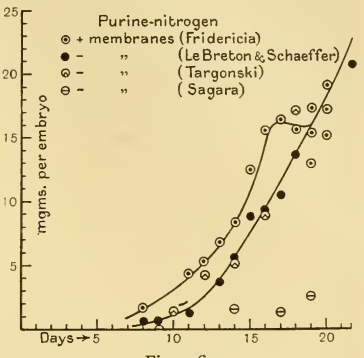


Fig. 346.

Targonski calculated the relation between the "anabolic" and the "catabolic" purines, in order to see how much purine nitrogen out of the total made each day was excreted into the allantoic liquid, and how much was stored up in the cells. He used his own figures for both sets of data, as follows:

| Day | Milligrams uric acid nitrogen per gram wet weight embryo in allantoic liquid | Milligrams purine nitrogen per gram wet weight embryo in embryonic body | Anabolic purine/ catabolic purine |
|---|---|---|---|
| 10 | 0·28 | 0·65 | 2·3 |
| 12 | 0·29 | 0·69 | 2·4 |
| 14 | 0·39 | 0·72 | 1·8 |
| 16 | 0·78 | 0·77 | 1·0 |
| 18 | 0·44 | 0·85 | 1·9 |

The ratio was, he concluded rather curiously, a constant, and meant that about 30 per cent. of the purine nitrogen formed on any one day is catabolic and 60 per cent. anabolic. In view of the considerable differences which have already been noted between the absolute values of different workers for uric acid production, it is difficult to decide whether these relations have any significance. Moreover, wet weight is obviously less satisfactory than dry weight for such a comparison. I therefore recalculated what may be called Targonski's ratio, using Murray's figures for dry weight, those of Fridericia and of LeBreton & Schaeffer for purine nitrogen, and my own for uric acid production. The justification for this is that all these investigators

used White Leghorn embryos. As Fig. 347 shows, the ratio, far from being, as Targonski thought, a constant, changes very markedly. Attention may first be concentrated on the curve obtained from LeBreton & Schaeffer's data, i.e. the curve for embryo alone, the membranes not being included. It begins at a very high value but immediately descends to reach a minimum on the 11th day, or, in other words, a condition when the excreted purine—the uric acid—somewhat exceeds the architecturally utilised purine. This minimum precisely corresponds, as is shown by the vertical line on the graph, with the point of maximum intensity of uric acid production, which has been already described, and which is pictured in Fig. 323. Later, Targonski's ratio rises again, and then falls slightly, although the uric acid excretion always exceeds the laying-up of purines in the cells. If now the Fridericia curve is considered, it will be seen that the ratio does not dip below the unity line till the 18th day, which shows that, if the purines in the membranes are taken into consideration, then the anabolic purines rather exceed the catabolic purine during most of development. The first trough is present, although shifted some 2 or 3 days forward, but the time of the subsequent peak is little changed. In short, Targonski's ratio varies during development with the intensity of protein metabolism, as we should expect from the fact that the main end-product of protein metabolism in the bird is a purine ring.

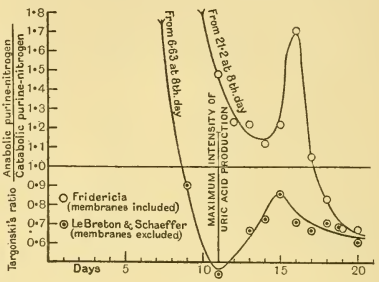



Fig. 347.

## 10·2. The Nucleoplasmatic Ratio

This expression was originally introduced by R. Hertwig, who in 1903 suggested that there was a definite relation between the mass of the nucleus and the mass of the cytoplasm. "The diminution of the nuclear mass seems, as Boveri has shown, to bring with it a diminution in cell size; the augmentation of the nuclear mass, according to Gerassimov, leads to augmentation of cell-size." The relation between the two was rapidly taken up by morphologists,

who evolved before long more or less quantitative methods of measuring it. As the basic nature of the work was always microscopical, these methods were restricted to the measurement of areas and surfaces. The school of Godlevski, which was pre-eminent in this field, used the method of drawing the cell and nucleus with a camera lucida, and then cutting out the drawing, pasting it on cardboard, and weighing the cardboard. Details of this method may be found in Godlevski's paper of 1921. It could not commend itself to anyone with a physico-chemical turn of mind, for it depended on the assumption that the surface area as seen microscopically bore a uniform relation to the mass, which, in view of the changing composition of nucleus and cytoplasm, may not be the case at all. Nevertheless, as a rough approximation the method was useful. Godlevski found great changes in the N.P.R. during maturation and segmentation in echinoderm eggs. Each stage has a characteristic N.P.R.:

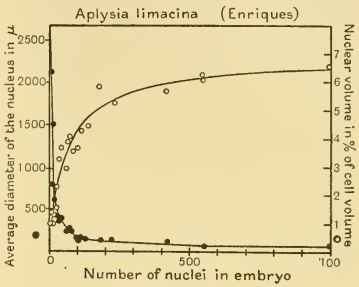



Fig. 348.

Table 164.

| | Total volume of nuclear material cubic μ | Nucleus volume | : | Cytoplasm volume |
|---|---|---|---|---|
| Unfertilised egg ... | 650 | 1 | : | 550 |
| Fertilised egg ... | 1300 | 1 | : | 275 |
| 2-cell stage ... | 1382 | 1 | : | 275 |
| 4-cell stage ... | 2084 | 1 | : | 177 |
| 32-cell stage ... | 19,938 | 1 | : | 18 |
| 64-cell stage ... | 30,262 | 1 | : | 12 |
| Blastula ... ... | 30,716 | 1 | : | 6 |

Thus as the nuclei become more numerous and individually smaller, the total mass of nuclear matter in the whole egg increases enormously. Other good series are those given by Enriques for *Aplysia* (Fig. 348), by Bury and by Erdmann. Similar work was done by Conklin. But to summarise all the researches which have been made on the morphological N.P.R. would serve no purpose, for they have already been reviewed in the monographs of Morgan, and of Fauré-Fremiet.

But a definite step forward was made by LeBreton & Schaeffer when they translated the older conception of N.P.R. into physico-chemical terms, and defined it as the expression:

$$\frac{\text{Purine nitrogen} \times 100}{\text{Total nitrogen} - \text{Purine nitrogen}}.$$

Two separate ideas seem to have been in the minds of the cytologists who dealt with N.P.R., firstly, that they were measuring simply cell and nuclear volume, and, secondly, that they were measuring somehow the amount of nuclear substance, or even nucleoprotein, present in the cell. LeBreton & Schaeffer's advance did not affect the first of these ideas, for microscopical measurement of N.P.R. still remains valuable cytologically, but it did supersede the second notion with a real hope of exactitude. It had always been obvious that nucleoprotein might be dispersed to some extent in the cytoplasm, and might not be all in the nucleus.[1]

One paper existed already in the literature in which definite information had been given about the "chemical" N.P.R., although its author did not perceive the full significance of his experiments. This was the memoir of Masing who estimated the nucleoprotein phosphorus in rabbit embryos according to the method of Plimmer & Scott, and obtained the following figures, which it is impossible to plot, in view of the imperfect characterisation of the material.

Table 165.

| | Nucleoprotein phosphorus in milligrams per 100 mgm. of total nitrogen |
|---|---|
| Whole rabbit embryos: | |
| 15 mm. ... ... ... ... | 5·8 |
| 21 gm. 4th week ... ... ... | 5·1 |
| 22·5 gm. 4th week ... ... ... | 4·85 |
| 28 gm. ... ... ... ... | 4·2 |
| 36 gm. 2 days before birth ... ... | 3·7 |
| 43 gm. Birth ... ... ... ... | 3·45 |
| After birth ... ... ... ... | 3·3 |
| Rabbit livers: | |
| Beginning of 4th week ... ... | 6·5 |
| Later stage ... ... ... ... | 5·8 |
| 1 day before birth ... ... ... | 5·1 |
| Birth ... ... ... ... ... | 4·85 |

From this it was evident that the nucleoprotein as related to the total protein of the body or organ diminished during development. The

[1] In recent times it has been convincingly shown that the greater part of the purine of adult muscle is present as (probably cytoplasmic) nucleotide and not as nucleoprotein. Studies on the N.P.R. must in future take this into account.

result of LeBreton & Schaeffer for the chick are shown in Fig. 348, from which it again appears that the chemical N.P.R. falls during development. Since Fridericia collected the same facts, it is possible to calculate a chemical N.P.R. from his figures, and Fig. 349 shows that a notable fall occurs in his data too.

LeBreton & Schaeffer also investigated the N.P.R. of the pig and the mouse (Fig. 351). They both descend in the same way as that for the chick. LeBreton & Schaeffer laid some stress, possibly

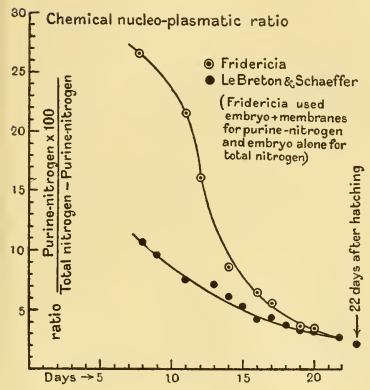



Fig. 349.

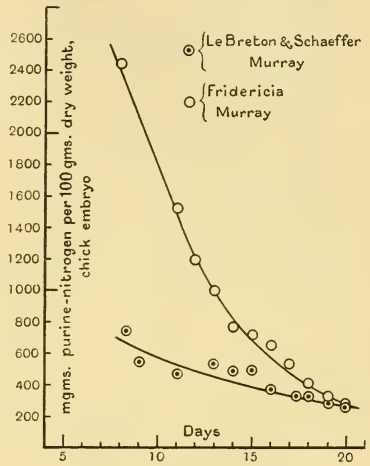



Fig. 350.

not quite justifiably, on the absolute value of the N.P.R. at the earliest stage, interpreting the fact that it fell from 12 in the case of the chick, and only from 7 in the case of the pig, to mean that the former was the seat of more intense transformations than the latter. In other words, they introduced a conception of potential, and suggested that perhaps differences in gestation time and life-span might be due to such differences, the chick, as it were, being suspended at a higher initial level than the pig, and correspondingly falling more rapidly to equilibrium. The forms of the curves led them to suggest that probably the most rapid fall would always be associated with the highest initial values: thus the chick's N.P.R.

descends from 11 to 4 in just over 10 days, but the pig's N.P.R. takes 70 days to descend from 8 to 4. "La hauteur du potentiel réalisé au moment de la 'mise en charge' des cellules de la blastula ne déterminerait-elle pas le régime de décharge ultérieurement réalisé pendant le développement?" said LeBreton & Schaeffer. "La décharge se ferait d'autant plus vite que la valeur initiale du N.P.R. serait plus élevée." It is as yet too early to say what fruit, if any, this interesting suggestion will bear, but it gains interest from Rubner's well-known table, which shows that the intensity of chemical transformations is greater the smaller the animal and the shorter its gestation time. This subject has already been discussed in some detail in Section 2.

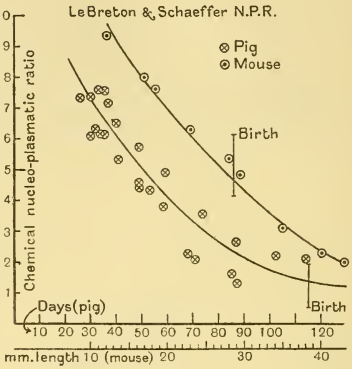



Fig. 351.

Table 166.

| | Duration of foetal life in days | Number of days required to double the birth weight | Nitrogen in grams fixed daily by 100 gm. nitrogen during this period | Initial height of N.P.R. |
|---|---|---|---|---|
| Man ... ... | 280 | 180 | 0·36 | — |
| Horse ... ... | 340 | 60 | 1·1 | — |
| Cow ... ... | 285 | 47 | 1·4 | — |
| Sheep ... ... | 150 | 13 | 4·4 | — |
| Pig ... ... | 118 | 15 | 4·7 | 7·5 |
| Guinea-pig ... | 67 | — | — | — |
| Dog ... ... | 63 | 8 | 7·4 | — |
| Cat ... ... | 56 | 9 | 7·3 | — |
| Rabbit ... ... | 28 | 6 | 11·0 | — |
| Mouse ... ... | 21 | — | — | 10·0 |
| Chick ... ... | 11 | — | — | 11·5 |

LeBreton & Schaeffer also pointed out the resemblance between the fall of N.P.R. and metabolic rate (compare Fig. 349 with Fig. 143).

From the figures now at their disposal LeBreton & Schaeffer were able to calculate the intensity of manufacture of purine nitrogen by the embryo. Smoothing their data for daily increments, they expressed

these in terms of 100 mgm. of purine nitrogen, and obtained the curve shown in Fig. 352. Fridericia's data were also treated in this way, but with quite different results, for, whereas the figures of LeBreton & Schaeffer give a markedly peaked curve at 12 days of development, those of Fridericia give an amorphous assemblage of points. LeBreton & Schaeffer pointed out that his figures included the purines of the membranes, and concluded that when their masking effect was removed by omitting them from the estimations—as they

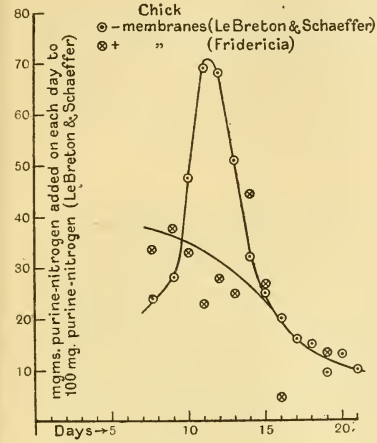



Fig. 352.

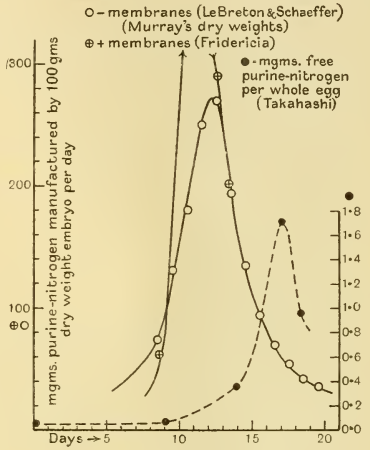



Fig. 353.

themselves had done—then a clear maximum of intensity of production of purine bases revealed itself. In order to have this important fact in a comparable form with other intensity curves, I recalculated the data in relation to dry weight (figures of Murray), with the result (shown in Fig. 353) that now both Fridericia's and LeBreton & Schaeffer's curves give a clear-cut peak about the 12th day. No one has had any explanation to offer of this peak in purine nitrogen production, but LeBreton & Schaeffer themselves hinted that it might be a curve of the same nature as the increment curve of an autocatalysed monomolecular reaction. They stated that their data for the pig and mouse embryo led to similar peaks.

As for the fall in N.P.R. itself, they mentioned the obvious relation between it and the fall in percentage growth-rate (see Section 2). The higher the growth-rate, the higher the N.P.R., i.e. the more purine nitrogen present per cent. of total nitrogen—but the connection between the two remains enigmatic. Defining further their notion, LeBreton & Schaeffer went on to say that with senescence the total nitrogen "grows" more rapidly than the purine nitrogen. They considered, further, that the N.P.R. would probably be found to rise from fertilisation to the blastula stage, and then to descend, but so far no determinations have been made which support or throw doubt on this view. LeBreton & Schaeffer themselves found the ripe eggs of the mackerel (*Scomber scombrus*) to have an N.P.R. of 1·5 and the adult liver cells one of 4·4, so that, if the latter had decreased from a much higher value, the former must have increased to it. More work along these lines would be very desirable.

## 10·3. Nuclein Synthesis in Developing Eggs

It has already been said that purine bases are undoubtedly synthesised by the developing chick embryo. The same holds for the embryo of the silkworm, if we may accept the early data of Tichomirov, who isolated 0·02 per cent. wet weight of total purine bases from the hibernating eggs, but over ten times as much, 0·23 per cent., from the fully developed embryos, of which about half was xanthine, and the rest hypoxanthine, guanine and adenine.

Similar results were obtained for the egg of the cod, *Gadus morrhua*, by Levene, although his methods were of doubtful validity.

| | Total purine bases % wet weight |
|---|---|
| Unfertilised eggs ... ... | 0·12 |
| 1 day's development ... | 2·16 |
| 11 days ... ... ... | 2·14 |
| 20 days ... ... ... | 3·75 |

Perhaps the increasing basic nitrogen found by Gortner in his trout and salamander eggs may indicate an increase in purine nitrogen. In the latter case especially there was a gain of nitrogen in the ether-insoluble-but-alcohol-soluble fraction of 4·1 mgm. nitrogen, or 0·7 per cent. of the total nitrogen, which he ascribed to the synthesis of purine and pyrimidine bases.

The only investigator who actually isolated purine bases from echinoderm eggs was Masing. In the unfertilised eggs of *Arbacia pustulosa*

he obtained 12·6 mgm. purine nitrogen from 2·96 gm. of dry substance, or 0·425 mgm. per cent., and from those in the morula stage 1·721 gm. gave him 7·56 mgm. nitrogen in the purine silver precipitate, or 0·439 mgm. per cent. In all cases 100 mgm. of total nitrogen contained 4·6 mgm. of purine nitrogen. In just the same way there was no change in the amount of nucleoprotein phosphorus up to the morula stage (see p. 1245). Masing's results were afterwards criticised by Robertson & Wasteneys on the ground that his material must have contained excess of spermatozoa, which are, of course, very rich in nucleoprotein, but this was not justifiable as Masing's unfertilised eggs gave as much purine nitrogen as later stages. Subsequent work by Needham & Needham on various invertebrate eggs strongly supported Masing, for the nucleoprotein phosphorus was found to be constant during development.

| | Nucleoprotein phosphorus mgm. per gm. dry weight | |
|---|---|---|
| | Before development | After development |
| Sand-dollar (*Dendraster excentricus*) | 2·22 | 2·54 |
| Starfish (*Patiria miniata*) | 1·04 | 1·56 |
| Sand-crab (*Emerita analoga*) | 1·26 | 1·59 |
| Brine-shrimp (*Artemia salina*) | 1·95 | 1·41 |

These facts are interesting in view of the great increase known to take place in the total quantity of microscopically visible nuclear matter during echinoderm development (see the figures of Godlevski given on p. 1151). All the early workers believed that nuclein synthesis occurred in echinoderm eggs, partly because, like Robertson, they were convinced that the choline of lecithin was the causative agent in cell-division, and the lecithin phosphorus must therefore be used in other ways, and partly because, like Loeb, they were impressed by the behaviour of the nucleoplasmatic ratio and identified nuclear chromatin with the oxidations of the cell on the one hand and with the supposedly autocatalytic character of growth on the other. The flaw in these arguments was the identification of nuclear material as seen through the microscope with nucleoprotein as measured chemically. With regard to the marine invertebrate eggs so far studied we must on the contrary picture an organisation of preformed nuclein into the chromatin of the nuclei rather than a chemical synthesis of it from other raw materials.[1]

[1] An apparent contradiction exists here. The histochemical method of Feulgen and Rossenbeck, which reveals the presence of nuclein, depends on a reaction of the aldehyde group of the hexose constituent with fuchsin sulphonic acid. J. Brachet has shown that

Yet if this is what happens in the simplest alecithic eggs, there is abundant evidence that it is not so in the most complicated ones. The preceding pages have demonstrated that the chick makes most of its nucleoprotein itself. We know practically nothing about this aspect of reptilian development, but the silkworm, as has been mentioned above, resembles the chick in nuclein synthesis. When, however, we pass to aquatic vertebrate eggs we find that the conditions are reversed, not because we know what occurs during development, but because notable quantities of nucleoprotein are found in the undeveloped eggs, in agreement with those of aquatic invertebrates and in contrast to terrestrial animals. Ichthulin itself, as has been described in Section 1·13 almost certainly contains no purine bases, but when the eggs have been worked up as a whole, investigators have found them (Levene & Mandel and Mandel & Levene on cod, Tschnernorutzki and Steudel & Takahashi on herring, and König & Grossfeld on herring, carp, cod, pike, and sturgeon eggs). Henze isolated over 1 per cent. of pentose from dried octopus eggs. Finally it is likely that nematode eggs contain large stores of nucleoprotein for Fauré-Fremiet found a good deal of the phosphorus to be combined in that way. We may summarise these facts in the following table:

| | Nuclein phosphorus | | |
|---|---|---|---|
| | In per cent. of the total phosphorus | | % of the final amount present at the beginning |
| | In the undeveloped egg | In the finished embryo | |
| AQUATIC. | | | |
| Sand-dollar (Needham & Needham) | 32·0 | 26·7 | 100·0 |
| Starfish ,, ,, | 15·6 | 25·4 | 61·0 |
| Gephyrean worm,, ,, | 15·8 | 54·1 | 34·6 |
| Sand-crab ,, ,, | 10·8 | 13·9 | 77·9 |
| Brine-shrimp ,, ,, | 37·9 | 27·4 | 100·0 |
| Sea-urchin (Masing) | — | — | 100·0 |
| Frog (Plimmer & Kaya) | 7·6 | 26·8 | 28·5 |
| TERRESTRIAL. | | | |
| Silkworm (Tichomirov) | By purine bases | | 9·5 |
| Hen (Plimmer & Scott) | 1·9 | 27·2 | 7·0 |

there is nothing like enough nucleic acid (as judged in this way) in the fertilised eggs of invertebrates to provide for the increase in nuclear material during development. If the nucleoprotein of the egg, however, contained pentose and not hexose, it would not give the Feulgen-Rossenbeck reaction and this, Brachet suggests, is the explanation of the discrepancy. Such an explanation is particularly plausible in view of the work of Calvery (see p. 1147).

Without formulating any definite generalisation it may be said that this association between nucleoprotein synthesis and terrestrial life is probably no coincidence. In Section 9·15 it was shown that embryos which develop in terrestrial, i.e. highly cleidoic, eggs excrete their waste nitrogen mainly in the form of uric acid, and this fact was advanced as an explanation for the existence of uricotelic metabolism in the birds, reptiles, and insects. Now if the uricotelic mechanism is essentially due to the presence of the enzyme uricoligase, which will synthesise the purine ring from lactic acid or some other three-carbon chain and ammonium carbonate or urea, it is interesting to find that wherever the uricotelic power exists, there also in the egg a notable synthesis of purine bases for chromatin exists, and where ammonia or urea are the end products of protein catabolism, there the egg is provided with sufficient or nearly sufficient purine bases at the beginning with which to construct its embryo. It is as if the synthesis of the purine ring were relatively difficult, and just as in the mammals there is a return to urea excretion after the uricotelic metabolism of the reptiles, so in the transition from amphibia to sauropsida, supplies of nuclein being no longer necessary in the egg there is a retention of this important material by the parent organism. It will be very interesting to see if future work supports the provisional rule that only uricotelic embryos synthesise nuclein.

As for the means by which this is accomplished nothing is known. Russo regarded his results as supporting the theory of arginine and histidine as precursors, but, as we have seen, Plimmer & Lowndes' results are in flat opposition to this view. Clementi has suggested that the protamines and histones, with their extremely high arginine-content, are in reality the intermediate products between the amino-acids of the yolk and the purine bases of the cellular nucleoproteins, but this is admittedly only a working hypothesis, and the subject is still one of the most obscure corners of the whole field.

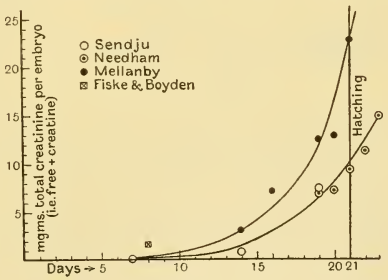



Fig. 354.

## 10·4. Creatinine, Creatine and Guanidine

The presence of creatine and creatinine in the unincubated avian egg was at one time a matter for dispute. In 1911 Salkovski and Kojo independently reported the existence of traces of creatinine in the fresh egg, although four years before Mellanby had completely failed to find any there. However, there has always been agreement that organised embryonic tissues contain creatine and creatinine. Krukenberg in 1880 detected the presence of the latter substance in the muscle tissue of foetal calves at various ages. Later, Mendel &

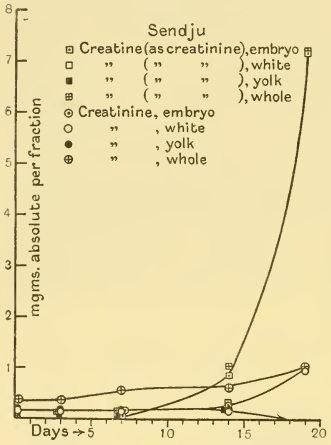



Fig. 355.

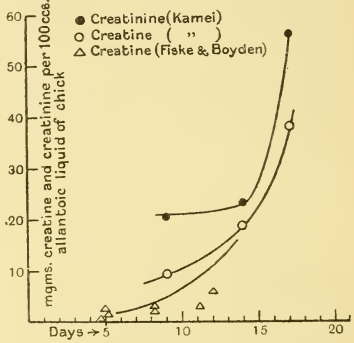



Fig. 356.

Leavenworth isolated 0·03 per cent. creatine from the muscles of a 265 mm. foetal pig—a low figure, the adult value being 0·45 per cent. Mellanby himself estimated the creatinine in the embryonic chick from the 14th day onwards, and some years later I obtained a series of figures parallel with his, but rather lower. These are shown in Fig. 354. There was little doubt but that the substance was present from the very beginning of development, and in 1924 Tiegs was able to demonstrate it by a colour test in the heart of the 4th-day embryo and in the general body tissue on the 5th day. Still later Fiske & Boyden reported a solitary value for the 8th day of development, also shown in Fig. 354.

The subject was gone into in more detail by Sendju, who estimated the creatine and creatinine in yolk, white and embryo. As Fig. 355 shows, the preformed creatinine never exceeds 1 mg. in the embryo, while the creatine rises to about 8 mg. The amounts contained in the yolk and white are at all times negligible, and it is evident that a vigorous synthesis must be proceeding.

The allantoic liquid, as has already been stated, contains creatine and creatinine. The concentrations of these substances rise steadily during development, as Fig. 356, plotted from the data of Fiske & Boyden and of Kamei, demonstrates.

As regards the origin of creatine and creatinine in the egg, nothing

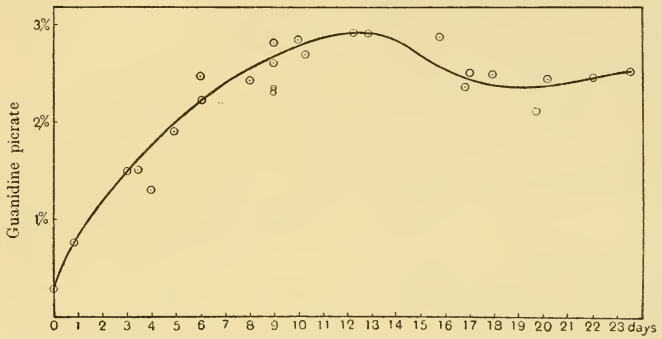



Fig. 357.

is known. The only attempt which has been made to find out is that of Burns, who in 1916 estimated the total guanidine in the hen's egg throughout incubation. The guanidine molecule is very resistant to oxidation, and Kutscher found that if a soluble protein is treated repeatedly with boiling calcium permanganate, a mixture of oxidation products is obtained from which much can be driven off as aldehydes, ammonia, carbon dioxide, etc., and from which the guanidine can be precipitated with picric acid. This was the method employed by Burns, who operated on whole eggs including everything except the shell. Fig. 357, taken from his paper, shows the grams of guanidine picrate found in per cent. of the egg-contents. There is clearly a steady increase in total guanidine picrate from 0·3 to 2·8 per cent. at the 12th day, after which there is a falling

off until the end of development. The formation of creatine cannot account for the decline in the curve during the latter part of development, for creatine and its anhydride were readily oxidised by the process used, and guanidine coming from them would be included in the figures for total guanidine. The total guanidine would have been expected to remain constant. Burns thought there were two possibilities, (1) that the configuration of the protein molecule of the chick might be more resistant to permanganate oxidation than the protein molecule of the yolk and white, or (2) that the guanidine precursor of creatine in the protein molecule may be partially destroyed by the oxidation process, just as enzyme action may sometimes split a protein through the guanidine group. Neither of these explanations is very satisfactory, for they both tend to throw doubt on the accuracy of the method as a test of the total guanidine present, and so to remove significance from the curve. A third possibility might be that after the mid-point of development the missing guanidine may be transformed into resistant rings such as pyrimidine or iminazol compounds. It is to be hoped that further work will be done on these obscure subjects.

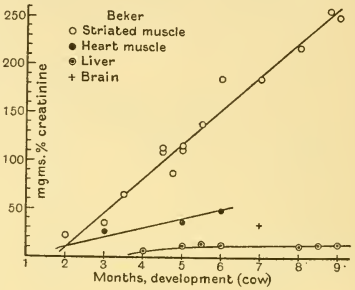



Fig. 358.

The best determinations of creatinine in developing organs of a mammalian foetus are those of Beker given in Fig. 358. The increase seems to be exceedingly regular.

Hunter has found 546 mgm. per cent. creatine in the muscle tissue of the ovoviviparous dogfish, *Squalus sucklii*, at a time when the muscles of its foetuses (7 cm. long) gave 460 mgm. per cent. Probably, therefore the elasmobranch foetuses accumulate the substance in much the same way as mammalian ones.

## SECTION 11

## FAT METABOLISM

### 11·1. Fat Metabolism of Avian Eggs

One of the first definite pieces of information acquired about the chemical changes during incubation was that the "fats" diminished in quantity. As early as 1846 Prévost & Morin reported that the total ether extract diminished from 10·72 per cent. of the egg-contents at the beginning of development to 9·82 per cent. on the 7th day, 9·48 per cent. on the 14th day and 5·68 per cent. on the 21st day. This was confirmed by Sacc in the following year, who made these interesting reflections: "Life is an ardent fire which needs nourishment incessantly; its activity is such that it will even devour its own hearth if it can find no other combustible. That is the reason why the same wisdom which we admire throughout nature has put at the disposal of the life in the egg this so abundant oil the destruction of which prevents that of the albumen. Without this oil of which the yolk is full (for it is in the yolk that the first traces of the embryo are formed) the albuminous matter would be burnt up by the oxygen of the air so that the development of the chicken could not go on".

Liebermann, in his important paper of 1888, decided that the fatty acids of the egg-contents were distributed thus: 40 per cent. oleic, 38 per cent. palmitic and 15 per cent. stearic. During incubation in one experiment the amount of "ether-soluble fatty substances" diminished from 5·401 gm. to 2·729 gm., and his general conclusion was that 2·672 gm. of fat were lost by an egg during development. This confirmed the older observations of Parke in 1866 and of Pott in 1879, but it is only since 1900 that data have been obtained of sufficient accuracy to allow of comparisons with those concerning protein and carbohydrate metabolism. Nevertheless Liebermann's figures were sufficiently definite to illuminate the subsequent respiratory researches of Bohr & Hasselbalch, and to lead to the conclusion that the expired carbon dioxide could be completely accounted for by the missing fat. It was here, of course, that the inaccuracy of the estimation methods for fat upset the calculations, and the quantitatively minor though very important participation of proteins and carbohydrates was overlooked.

Among the later workers, Tangl & von Mituch and Iljin simply estimated the amount of fatty acids in the whole egg before and after development.

Table 167.

| | Grams of fat per egg | | | |
|---|---|---|---|---|
| | Before | After | Loss | |
| Tangl & von Mituch ... | 4·76 | 2·90 | 1·80 | |
| | 4·84 | 2·69 | 2·15 | |
| | 5·00 | 2·92 | 2·18 | |
| | 6·33 | 4·19 | 2·14 | |
| | 6·11 | 3·90 | 2·21 | |
| | 5·88 | 3·65 | 2·23 | |
| | | Average ... | 2·11 | |
| Iljin ... ... ... | 6·97 | 2·87 | 4·10 | Outside limits |
| | 3·37 | 0·97 | 2·40 | |
| | | Average... | 3·25 − 1·20 = 2·05 | |
| Cahn ... ... ... | 4·5 | 3·13 (1·87 in yolk, 1·26 in embryo) | 1·37 (average) | |

Iljin's figures are not comparable with those of Tangl and of Tangl & von Mituch, for he worked only on the yolk, and, though the exclusion of the egg-white does not matter, as there is practically no fat in it, yet the exclusion of the chick at the end of development would make a considerable difference. Judging from Murray's figures we might put its fat content at 1·2 gm., and this subtracted from Iljin's figure would make it agree with the rest. Tangl & von Mituch divided the fatty acids as follows: of the original 5·68 gm. in the average egg, 1·59 gm. went into the embryo, i.e. 28·0 per cent. of the original amount, 1·79 gm. remained in the yolk at the end of incubation, i.e. 31·5 per cent., leaving 2·11 gm. fat burnt, or 40·5 per cent. Sakuragi and Idzumi later studied the fat loss by the hen's egg in detail, both using a modified Kumagawa-Suto technique. Their results are plotted on Fig. 359, from which it can be seen that the utilisation of fatty acids follows a regular curve, descending more rapidly towards the end than towards the beginning of incubation. On the same graph are included the figures of Eaves; Murray, and some already mentioned, and from the whole group a notable measure of agreement appears. It is difficult to draw exact conclusions from a curve which has been constructed from the data of so many different investigators, but it is certainly interesting to plot the milligrams of fatty acid disappearing each day from the egg, and

this can easily be done by calculating the mean daily decrement. Such a curve is shown in Fig. 360, and on the same graph appears

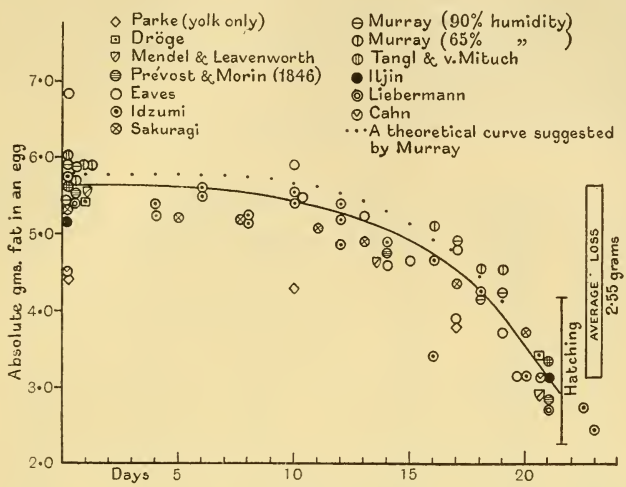



Fig. 359.

a curve calculated by Murray from the carbon dioxide output, assuming that this was derived entirely from fat oxidation. There is evidently a discrepancy, for from the 7th to the 14th day the fat lost, as determined by the averaged chemical analyses, is in excess of that lost as determined from the carbon dioxide output, even supposing that all the carbon dioxide was derived from fat, which is not true. The figures of Bohr & Hasselbalch for carbon dioxide output would give an even worse divergence, for during this particular period they were lower than those of Murray. The explanation for this missing

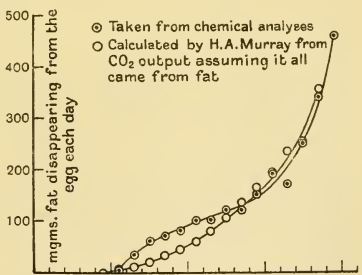



Fig. 360.

fat must be that during that period it is used for other purposes than combustion. If the discrepancy is not simply an error due to imperfect technique, there is every probability that at this period the missing fat is transformed into carbohydrate, for a coincident increase in total carbohydrate occurs. This subject has been discussed in the Section on carbohydrate metabolism, to which reference may be made (pp. 1016–1018).

Table 168.

| | Grams of fat in whole egg | Intervals of days | Milligrams of fat utilised per day | | Milligrams of fat utilised per day calculated by Murray from carbon dioxide output |
|---|---|---|---|---|---|
| | | | Experimental | Smoothed | |
| Day 0 | 5·6 | 0–1 | 0 | 0 | 9 |
| 1 | 5·6 | 1–2 | 0 | 0 | 0 |
| 2 | 5·6 | 2–3 | 0 | 0 | 0 |
| 3 | 5·6 | 3–4 | 0 | 0 | 0 |
| 4 | 5·6 | 4–5 | 0 | 0 | 3 |
| 5 | 5·6 | 5–6 | 0 | 0 | 6 |
| 6 | 5·6 | 6–7 | 0 | 0 | 11 |
| 7 | 5·6 | 7–8 | 20 | 20 | 20 |
| 8 | 5·58 | 8–9 | 50 | 50 | 33 |
| 9 | 5·53 | 9–10 | 80 | 80 | 45 |
| 10 | 5·45 | 10–11 | 100 | 94 | 60 |
| 11 | 5·35 | 11–12 | 100 | 105 | 80 |
| 12 | 5·25 | 12–13 | 120 | 116 | 105 |
| 13 | 5·13 | 13–14 | 120 | 127 | 132 |
| 14 | 5·01 | 14–15 | 150 | 145 | 164 |
| 15 | 4·86 | 15–16 | 190 | 168 | 198 |
| 16 | 4·67 | 16–17 | 170 | 205 | 236 |
| 17 | 4·50 | 17–18 | 250 | 250 | 253 |
| 18 | 4·25 | 18–19 | 340 | 331 | 359 |
| 19 | 3·91 | 19–20 | 460 | 460 | — |
| 20 | 3·45 | — | — | — | — |
| | | | 2150 | | |

We may now consider the growth of the fatty substances in the embryonic body. As Fig. 361 shows, there is a considerable divergence in the absolute value of the figures, but this is no doubt due to the various methods used, as will be shown below. One point of importance, however, emerges from this group of curves, namely, the inflection which they all possess about the 14th day. This corresponds with all that we know about the absorption intensity of fatty substances by the embryo (see Fig. 251), and supports the conclusion that there is a definite awakening of fat metabolism towards the end of development. Another closely related fact is the percentage constitution of the embryo, shown in Fig. 244, which indicates an

important rise in the fat during the week before hatching—and it will be remembered that analyses of other embryos than the chick demonstrated fragments of the same set of curves. Facts of simple observation, too, confirm the enhanced activity of fat metabolism at the end of development. Metzner observed fat globules in the liver of the chick on the 12th day, not before, after which time they rapidly increased in number and in size, and this was but a confirmation of the earlier work of E. H. Weber. Nordmann, again found no fat in explanted liver cells from 8th or 9th day embryos but an abundance in those from the last week of incubation. He noted that fat added to the medium on which the former were growing would pass into the cells. Virchow, studying the yolk-sac of the chick histologically, observed fat drops after the 10th day, but never before. On the quantitative side, Riddle's estimations of fat in the yolk towards the end of incubation, which will be mentioned again later, show a preferential utilisation of fatty acids by the chick embryo.

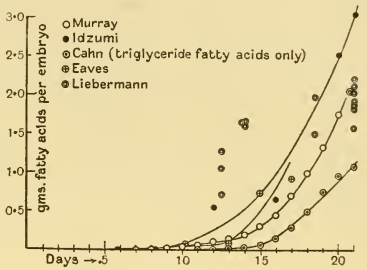


Fig. 361.

Important information on these questions has been gained by the use of the dye Sudan III, which attaches itself to fat molecules and indicates their presence. Sitovski was the first to make use of this substance, and by feeding it to moths was able to obtain their eggs deeply pigmented. Riddle then independently fed Sudan III to laying hens (3 to 25 mgm. of the dye per hen per day), and observed that the yolks of their eggs became red, or rather, that the yellow yolk became red, the rings of white yolk and the latebra remaining pale yellow, or becoming very pale pink, thus according with the chemical analyses (see p. 286). Riddle succeeded in pigmenting the eggs of turtles in the same way, and since that time his experiments on fowls have been confirmed by many investigators (e.g. Halnan). Gage & Gage were the next to go into the matter, and, feeding hens on the dye, incubated the resulting eggs. "As the yolk softens during the process of incubation", they said, "the layered (pink and yellow) mass becomes homogeneous and of a uniform pink. This is marked from

the 3rd day onwards. For the first 10 days the transparent embryo shows no sign of the colour, but as soon as the chick begins to deposit fat, at the 17th day of incubation, a minute mass of fat lying in the loose connective tissue between the leg and the abdomen was found with the characteristic pink colour which deposited fat takes in adults fed with the stain. At this time the yolk-mass is of a nearly uniform dark red and almost enclosed within the body." These beautiful observations fit in exactly with the chemical evidence, and are in good agreement with the rest of our knowledge about fat metabolism. They were subsequently repeated and confirmed by Gage & Fish, but work is still required, for Halnan in some unpublished experiments has failed to obtain such clear-cut results as were reported by Gage & Gage. Cross and Rogers reported some curious facts relating to Sudan III. The usual banded appearance of the yolk disappeared by the 5th day of development, and shortly afterwards "the albumen near the embryo" took on a pink colour. On separating by coagulation *in situ*, Cross found the following distribution of fat:

| | Neutral fat % dry weight |
|---|---|
| Yolk ... ... ... | 63·4 |
| White albumen ... ... | 0·2 |
| Pink albumen ... ... | 55·0 |

For other work on Sudan III and the fat metabolism of the reproductive system see Riddle's paper of 1910.

The technique used in the greater part of the researches hitherto referred to was crude, though perhaps uniform as far as it went. Thus Murray used the term "fat" to designate the extract obtained after washing the ground-up dried tissue with a mixture of equal parts of alcohol and ether followed by a 24-hour extraction with re-distilled anhydrous ether in a Soxhlet extractor, the material being re-ground with sand half-way through the process, and the extract being finally dried to constant weight. Such an extract would include a large number of substances besides pure triglycerides or neutral fats, and it was clearly necessary to make a more detailed investigation. This was done by Cahn, who obtained experimentally the amount of total fatty acids, and subtracted from this the amount associated with the lipoid phosphorus on the one hand, and the amount present as cholesterol esters on the other hand. He differed completely from Murray in finding that, although the total fatty

acids and the total alcohol-ether extract rose in grams per cent. wet weight, the latter remained quite constant in per cent. dry weight. On the other hand, the total fatty acids did show a rise, similar to Murray's. Fig. 362 shows this, but it only means that some differences between the techniques used by Murray and by Cahn, which, owing to the lack of detailed information in their papers, we cannot exactly define, caused discrepancies in the earlier stages. In any case, the

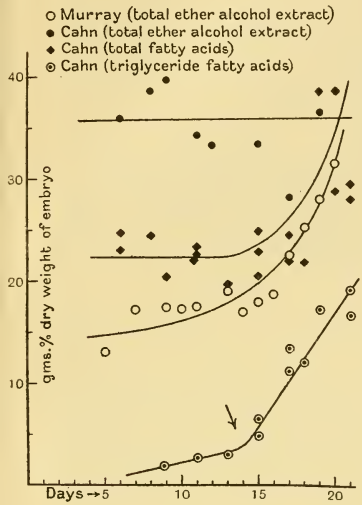



Fig. 362.

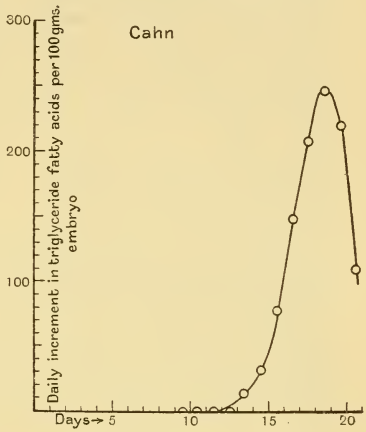



Fig. 363.

total alcohol-ether extract is not an entity of great interest. Cahn's figures for triglyceride fatty acids are shown in Fig. 362, expressed in per cent. of the dry weight of the embryo. Here the rise is undoubted, and it is significant that the usual 14th day inflection appears on the curve.

If the daily increment is plotted, an interesting bell-shaped curve appears. As Fig. 363 shows, 100 gm. add on to themselves more and more fatty acids (triglycerides) until the 19th day, after which there is a slackening off. This agrees well with the absorption intensity curve for fat as shown in Fig. 251. Cahn's interesting com-

parisons between the behaviour of the true fatty acids and the lipoids, and his calculations of tissue constants will be discussed more easily in the Section on lipoids and sterols. He also calculated the percentage growth-rate of the fatty acids of the triglycerides, obtaining a curious result which he did not explain. The curves plotted in Fig. 364 are, firstly, the percentage growth-rate (Minot) of the wet weight of the embryo, secondly, a composite curve for the growth-rate of dry weight, calorific value, total carbohydrate, coagulable protein, and total ether-alcohol extract, all taken from the data of Murray and Needham. The dry weight growth-rate exhibits a plateau between the 10th and 15th days because the dry weight is then most rapidly increasing, but Cahn's curve for growth-rate of triglyceride fatty acids has not a plateau, but a peak. On the other hand, his percentage growth-rate curves for lipoid phosphorus and for cholesterol follow approximately the usual course.

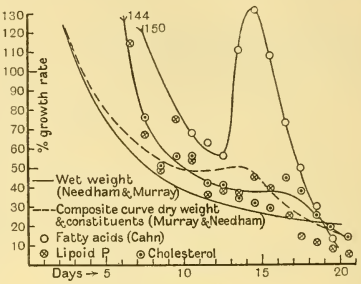



Fig. 364.

Something must next be said about the variations in the nature of the fatty acids in the egg at the different stages of development. Mottram showed in 1913 that the mean iodine value of the fatty acids in the hen's egg was constant, and did not vary greatly. He studied the individual variations, and the effect of incubation on infertile eggs, and his results have already been discussed in Section 1. The first week of incubation, he found, affected the iodine value but little in the fertile egg, but he sometimes got evidence of a slight rise, thus:

| Day | Iodine value |
|---|---|
| 0 | 81 |
| 4 | 83 |
| 8 | 88 |
| 12 | 80 |
| 16 | 84 |
| 19 | 86 |

He was inclined to regard his results as demonstrating the possibility of desaturation outside the liver cells. More interesting were the experiments of Eaves, who estimated the iodine value of the fatty

acids of the chick embryo and the rest of the egg throughout development. Her results are plotted in Fig. 365, from which it appears that there is little change during the first 10 days of development, but that after that time the iodine value of the fatty acids in the chick rises, while that of the fatty acids in the remainder of the egg falls. This decrease in the iodine value of the yolk-fat during development is highly suggestive of a primary absorption of the less saturated fats with a subsequent equal absorption of saturated and unsaturated fat. The increase in the iodine value of the embryonic fatty acids points to an absorption of the more desaturated fatty acids, and suggests that in the early stages the chick embryo does not possess the power of performing the desaturation. If the embryonic liver was incapable of desaturating fatty acids until, say, the 10th day of development, it might be supposed that the unsaturated fatty acids would be preferentially absorbed, as the saturated ones would be of no use to the embryo. However, no *a priori* arguments are here of importance, and I accordingly arranged a series of experiments to test the hypothesis. An aqueous emulsion of embryonic tissues was mixed with the corresponding yolk and vigorously shaken, after which it was allowed to stand anaerobically under toluene for 5 days at 37°. The results were as follows (fatty acids being separated by the Mottram-Lemeland technique):

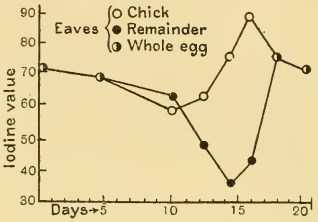


Fig. 365.

| | Iodine value | |
|---|---|---|
| | Before experiment | After experiment |
| 6-day yolk alone ... ... ... ... | 76·0 | 66·8 |
| 6-day embryo and 6-day yolk ... ... | 69·8 | 66·4 |
| 12-day embryo and 12-day yolk ... ... | 72·8 | 91·6 |
| 19-day embryo and 19-day yolk ... ... | 61·2 | 80·0 |

Thus where the 12th and 19th day embryonic cells were present there was a very marked desaturation occurring, but not in the case of the 6th day embryo, nor the 6th day yolk alone. If this set of experiments should be confirmed, it will show that in the earlier stages the embryo does not possess the power of desaturating the fatty acids it absorbs from the yolk. It is therefore forced to use the unsaturated

ones, and possibly, as these are not present in great quantity, it is thus prevented from devoting much fat to combustion.

In a previous discussion of protein metabolism, the work of Riddle on the yolk-contents in the latter half of incubation was cited, and it is equally relevant here, for he determined the percentage (dry weight) of neutral fat in it from the 12th day onwards, using the method of Koch. His data, which are plotted in Fig. 366, demonstrate that the percentage of fat in the yolk-sac falls towards the end of development, just as the percentage of protein rises, indicating a pre-

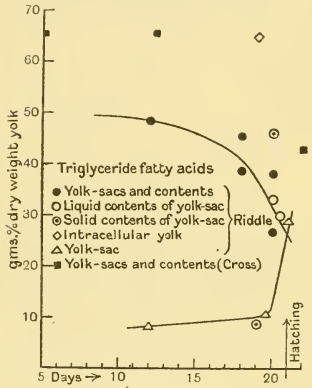



Fig. 366.

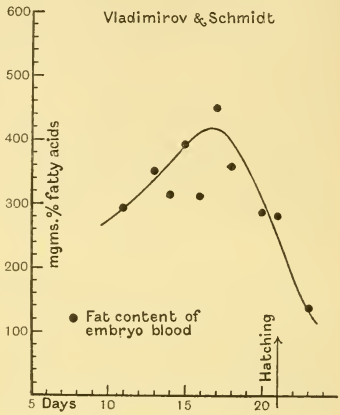



Fig. 367.

ferential absorption of the former over the latter, and bearing out the absorption intensity curves shown in Fig. 251. The rest of the information is more difficult to understand, and concerns special fractions of yolk; thus the intracellular yolk (yolk-masses in the wall of the yolk-sac) seems to have a high fat concentration, and the solid masses in the body of the yolk itself a variable one. As for the yolk-sac, its fat-content rises greatly in the last few days of development. We shall see later that this preferential absorption of fatty acids is accompanied by a preferential absorption of phosphatides. This picture of the awakening of fat metabolism in the last days of incubation is to some extent mirrored in the results of Vladimirov & Schmidt on the blood fat of the embryo. As Fig. 367 shows, it

rises to a peak on the 18th day of development, and then falls without a break at hatching. This peak corresponds well enough with the peak in the daily increment of fatty acids found by Cahn (Fig. 363), and occurs at the time when the absorption intensity of fatty acids (Fig. 251) is rising away from its 14th day trough, and taking the place of the protein absorption curve, which is descending. It must be admitted, however, that the individual variations in the curve of Vladimirov & Schmidt are considerable, and it would be worth while to repeat their measurements, using perhaps a better method (they employed that of Bang).

## 11·2. Fat Metabolism of Reptilian Eggs

The fat metabolism of the reptile egg has only once been investigated—by Karashima in 1929, who worked with the turtle, *Thalassochelys corticata*. He found a diminution of some 30 per cent., rather low for a terrestrial egg, as the following table shows:

| Days of development | Grams fatty acid per egg |
|---|---|
| 0 | 1·64 |
| 15 | 1·68 |
| 30 | 1·63 |
| 45 | 1·36 |
| Hatched | 1·12 |

Karashima also investigated the percentage of free fatty acids, water-soluble and water-insoluble volatile fatty acids, etc., in the egg-fat at the various stages, but these showed no definite changes: the first-named remaining at about 20 per cent. of the total fraction, the second at about 0·9 per cent. and the third at about 0·7 per cent. On the 15th day, however, there was a marked rise in the free fatty acids, no doubt to be interpreted as due to the action of a lipase, since the total fatty acids remained constant.

## 11·3. Fat Metabolism of Amphibian Eggs

The fat metabolism of the developing amphibian egg has been a subject of some difference of opinion between investigators. Parnas & Krasinska measured the total fatty acid content of frog embryos (*Rana temporaria*) using the Liebermann-Kumagawa-Suto method. As Table 169 shows, they found just as much at the time of hatching as at the beginning of development, and they therefore concluded that none was used as a source of energy. They did, however, find

Table 169. *Fat Metabolism of the Amphibian Embryo* (Frog, *Rana temporaria*).

| Investigators | Total fatty acids: Beginning of development: Milligrams per egg | Beginning of development: % wet weight | Beginning of development: % dry weight | Hatching: Milligrams per egg + embryo | Hatching: % wet weight | Hatching: % dry weight | End of Yolk-sac stage: Milligrams per larva | End of Yolk-sac stage: % wet weight | End of Yolk-sac stage: % dry weight | Milligrams lost: Phase 1 | Milligrams lost: Phase 2 | Milligrams lost: Whole | Iodine value before | Iodine value after | Molecular weight before | Molecular weight after |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parnas & Krasinska ... ... | 0·283 | 10·90 | — | 0·283 | — | — | — | — | — | 0 | — | — | 106·4 | — | — | — |
| ,, ,, ... ... | 0·336 | 12·90 | — | 0·335 | — | — | — | — | — | — | — | — | — | 104·0 | — | — |
| ,, ,, | 0·322 | 12·30 | — | 0·343 | — | — | — | — | — | — | — | — | — | — | 362 | 364 |
| Terroine & Barthélemy ... | — | 10·87 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Fauré-Fremiet & Dragoiu ... | 0·413 | 10·14 | 23·93 | 0·300 | 6·63 | — | — | — | — | 0·113 | — | — | 123 | 113 | — | — |
| Bialascewicz & Mincóvna: (Kumagawa-Suto method) | 0·325 | — | — | 0·336 | — | — | 0 | — | — | 0 | — | — | — | — | — | — |
| (Bang method) ... ... | 0·409 | — | — | 0·454 | — | — | 0·286 | — | — | 0 | 0·192 | 0·192 | — | — | — | — |
| ,, ,, ... ... | — | — | — | — | — | — | 0·335 | — | — | — | — | — | — | — | — | — |
| ,, ,, ... ... | — | — | — | — | — | — | 0·162 | — | — | — | — | — | — | — | — | — |
| Barthélemy & Bonnet ... | 0·280 | — | 12·65 | — | — | — | 0·178 | — | 15·1* | — | — | 0·102 | — | — | — | — |
| Fauré-Fremiet & du Streel ... | — | 3·31? | — | — | 6·65 | — | — | — | — | — | — | — | — | | — | — |
| Average... | 0·337 | | | | | | | | | Average... | | 0·140 | | | | |

* Up to disappearance of external gills, not quite to the end of the yolk-sac period.

a decrease in lipoid phosphorus, as will be described in the following section, so that it seemed as if the amount of fatty acids in triglyceride form must even rise rather than fall. Parnas & Krasinska did not pursue their investigations into the free-swimming larval stage. The work of Fauré-Fremiet & Dragoiu paralleled that of Parnas &

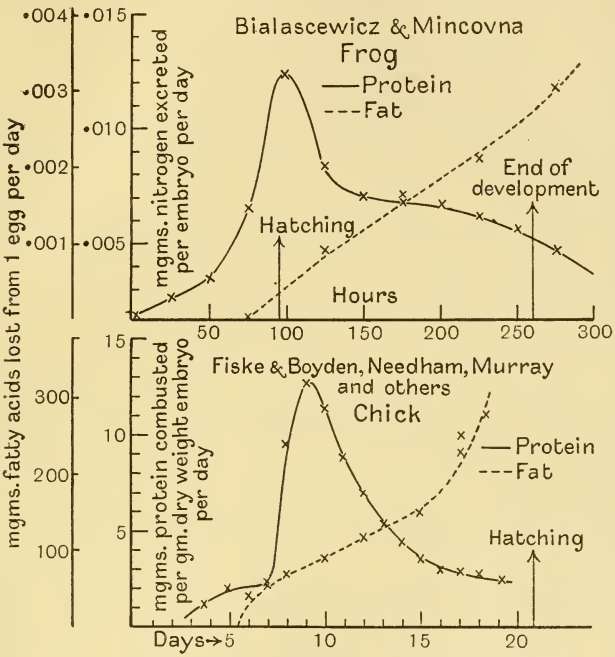



Fig. 368.

Krasinska, in that only the pre-natal period was considered, but it led to diametrically opposite results for they found a diminution in total fatty acids amounting to 0·113 mgm. per embryo. Two other researches, however, followed the development up to the end of the yolk-sac. Bialascewicz & Mincóvna measured the fatty acids at the end of each period, and for the first of the two they confirmed Parnas & Krasinska, observing no change, or even an increase, in the fatty

acid content up to hatching. Bialascewicz & Mincóvna used two methods, that of Kumagawa-Suto and that of Bang, obtaining substantially the same results with each, while Fauré-Fremiet & Dragoiu used that of Kumagawa-Suto alone. Still later Barthélemy & Bonnet fully confirmed the loss of fatty acids over the whole of development found by Bialascewicz & Mincóvna, noting a loss of 0·102 mgm. per embryo, instead of 0·192. They did not, however, make any estimations on the just-hatched embryos, and so had nothing to say on the difference between Fauré-Fremiet & Dragoiu and the other workers. Possibly the breed of frog used may account for this. In any case we shall assume here that practically no fat disappears from the amphibian egg before hatching, but that afterwards a considerable utilisation takes place. The rate at which the fatty acids disappear was sketched out by Bialascewicz & Mincóvna in a few experiments, and is shown in Fig. 368, where in the upper graph the amount of fatty acids disappearing from one larva per day is plotted against the time. The curve rises more or less steadily. For the sake of comparison the curve of protein utilisation established by Bialascewicz & Mincóvna is placed beside it, and we thus see how at a certain point the combustion of protein attains a maximum and afterwards falls away, giving place to the catabolism of fatty acids. Below is placed a composite graph showing the utilisation of protein and fat by the chick embryo (taken from Figs. 325 and 360). The likeness between the two pictures is quite striking. Not only do both embryos exhibit a peak of protein catabolism, but the peak comes at approximately the same epoch of development, and this although the frog derives 70 per cent. of its waste energy from protein and the chick only 5 per cent., and although the time of hatching in the two organisms is so completely different. For various reasons, which have already been stated, we cannot add curves for carbohydrate catabolism to the pictures, nor have any other embryos been investigated in sufficient detail to permit of parallel graphs being drawn for them. In the case of mammalian and selachian embryos, indeed, it is possible that we have already the descending limb of the peaked protein catabolism curve (see p. 1117), and Hayes' account of the constitution of the embryo of the Atlantic salmon (*Salmo salar*), which shows a rising fat-content up to the 106th day from fertilisation, followed by a fall, may indicate a late onset of fat catabolism in that case also.

But to return to the fat metabolism of the frog embryo, the relations

between fat and nitrogen depicted in Fig. 368 could be expressed in terms of a ratio. This Bialascewicz & Mincóvna did, as follows:

| Hours from fertilisation | Milligrams fatty acid in one larva/ Milligrams nitrogen in one larva |
|---|---|
| 0 | 3·61 |
| 72 | 3·96 |
| 96 | 4·26 |
| 140 | 4·13 |
| 143 | 4·07 |
| 164 | 3·49 |
| 239 | 2·86 |
| 284 | 2·50 |

The ratio tends to rise until hatching because the denominator is decreasing while the numerator is remaining constant, but in the later stages the numerator falls more rapidly than the denominator, so that the ratio falls.

Fauré-Fremiet & Dragoiu studied the iodine value of the fatty acids of the frog's egg, as did Parnas & Krasinska. But again there was a certain contradiction, for whereas the Polish investigators only found a diminution of 2 units, the French ones found a diminution of 10 units. Fauré-Fremiet & Dragoiu calculated that at the beginning of development 80·81 per cent. of the total fatty acids were unsaturated (1 double bond) and at the end (hatching) only 74·14 per cent. By multiplying the lipoid phosphorus by the coefficient 25·75, the percentages of phosphatide fatty acids were found, and this fell from 25·13 to 20·0 per cent. One embryo lost up to hatching, they found, 0·192 mgm. of unsaturated acids, and 0·06 mgm. of phosphatide fatty acid, but it must be remembered that none of the other workers found any loss of fat before hatching.

Barthélemy & Bonnet carried out their experiments at different temperatures, in order to see whether the rapidity of development would exercise any influence upon the amount of fatty acids lost throughout the whole period. Their results were as follows:

| Temperature (°) | Fatty acids lost in % of initial fatty acids | Loss of fat/ Loss of nitrogen |
|---|---|---|
| 8 | 48 | 0·75 |
| 10 | 32 | 0·73 |
| 14 | 41 | 0·75 |
| 21 | 38 | 0·75 |

showing that, however much the development may be speeded up, or damped down, the same amount of fatty acids have to be combusted. Moreover, the ratio of fat lost to nitrogen lost is identical, and compares in an interesting way with the ratio of Bialascewicz & Mincóvna for

the embryonic or larval body itself. This indicates that relatively much more protein is combusted than fat, as has been shown in Table 126.

So far the eggs of the anuran branch of amphibia have alone been considered, but one chemical investigation exists which deals with those of a urodele, the giant salamander, *Cryptobranchus allegheniensis*. This is that of Gortner, to which attention has already been given in Section 9·9. We have seen above that the general consensus of opinion leads to the view that no fatty acids are lost from the egg of *Rana temporaria* up to hatching. But, if Table 169 is carefully examined, it will be seen that Bialascewicz & Mincóvna observed an actual increase in fatty acids during the pre-natal phase. The increase was not great, being about 11 per cent., but, if they were there dealing with a real phenomenon, it was interesting, for a synthesis of fatty acids was found by Gortner to be important in the salamander egg. Gortner's facts were as follows (he did not follow the development further than hatching):

Table 170.

| | Weight in milligrams absolute | | | % dry weight | | |
|---|---|---|---|---|---|---|
| | 1 egg | 1 larva | Change | Eggs | Larvae | Change |
| Dry weight ... ... | 58·25 | 57·28 | −0·97 | — | — | −1·66 |
| Total ether extract ... | 11·18 | 12·75 | +1·57 | 19·19 | 22·25 | +3·06 |
| Ether-insoluble but alcohol-soluble ... ... | 6·63 | 6·18 | −0·45 | 11·38 | 10·78 | −0·60 |
| Protein ... ... ... | 40·26 | 38·28 | −1·98 | 69·10 | 66·82 | −2·28 |

The loss of 1·66 per cent. of the dry weight was due to combustions to carbon dioxide and water. "Accompanying this loss in weight", said Gortner, "there is a very marked gain of fats equal to 3·06 per cent. of the egg weight and to an increase of 14 per cent. of the fat already present in the egg." Gortner meant by "fat" the total ether extract dried to constant weight, in which a number of other substances would be included. It was not long before the fact of fat synthesis in this egg was confirmed, by the aid of an accurate method. McClendon in the following year applied the Kumagawa-Suto method to the same material. He observed a numerically identical loss of dry weight between fertilisation and hatching, and the figures for total extract compared as follows:

| | Total ether- and alcohol-soluble substances % of the dry weight | | Increase (%) | |
|---|---|---|---|---|
| | Eggs | Larvae | Dry weight | Initial |
| Gortner | 30·57 | 33·03 | 2·46 | 6·9 |
| McClendon | 35·0 | 37·8 | 2·8 | 8·0 |

The determination of the non-volatile fatty acids showed them to amount to precisely 50 per cent. of the total extract in each case, so that the increase in them corresponded with the increase in the total extracts. McClendon explained the slight lowness of Gortner's figures as compared with his own by referring to the extreme difficulty of removing by extraction the last traces of phosphatides from yolk, owing to their association with vitellin. McClendon found the Molisch reaction negative on the whole egg, and could observe no reduction of copper after total hydrolysis (experimental details not given), so he concluded that no carbohydrate groups were present, and that the increase in fatty acids must be due to the destruction of protein molecules. The evidence for this view was insufficient.

Some histochemical investigations have been made on the developing amphibian egg. The work of Konopacka and of Konopacki & Konopacka is especially detailed, and Hibbard has given an exhaustive study of the histochemistry of the development of the anuran, *Discoglossus pictus*. Unfortunately, as I have pointed out before, we have no guarantee that the substances which the histochemical worker studies are the same as those which we analyse and measure by purely chemical methods, though they may be, and usually are, called by the same names. For this reason it is very difficult to know what emphasis to lay on the findings of these workers. Thus Konopacki & Konopacka, who worked with the embryos of *Rana temporaria*, reported a disappearance of "fat" during the segmentation stages, and concluded that "the intensity of metabolism is parallel to the morphogenetic changes", though how this result could be arrived at from the study of stained sections is not clear. But Konopacki & Konopacka had less to say about neutral fats than about lipoids, as the Flemming-Ciaccio method is supposed only to reveal the presence of the latter. However, "in the early stages of development", says Konopacka, "the fatty acids are utilised, for the number of droplets staining with osmic acid are more numerous and larger than those which stain with Sudan III after fixation according to Ciaccio's technique. In later (post-gastrula) stages, however, the two kinds of droplets are of the same size, and eventually the 'lipoid' droplets disappear completely, once the structure of an organ is determined". Hibbard says that, during the early development of *Discoglossus*, there is little change in the distribution or appearance of the fat globules, but just before hatching they begin to increase in number

and size, reaching a maximum at the time when the external gills are half covered over by the operculum, and afterwards falling away again. By the time the tadpole has reached a length of 17 mm. no fat droplets are to be found in the cells. She concluded that some of the fatty acids absorbed from the yolk were utilised in the cells of the tissues, but that most of them were eventually transferred to stock the newly formed liver cells.

Among the histochemical researches, one of the most interesting is that of Abe, who began by the not very hopeful statement that "the anuran yolk is a complicated lipoid-protein mixture containing phosphoprotein and iron", but went on to show that the utilisation of the substances in it could not be going on at a uniform rate, because in the early stages eosin stains the yolk light red or pink, later dark red, and finally deep violet-red. No direct physico-chemical meaning can as yet be attached to this, but it gives a glimpse of the unequal utilisation of the yolk constituents which we have been discussing.

## 11·4. Fat Metabolism of Selachian Eggs

We can now consider the events which take place in the fish egg with respect to fatty acids during its development, and we shall begin with the ovoviviparous selachians. In these fishes peculiar relations seem to exist between the liver of the "pregnant" fish and the embryos within their transparent envelopes in its uterus. Lo Bianco was the first to notice that whenever embryos were present the size of the liver was increased, and this observation was confirmed by Polimanti, who gave the following figures:

Table 171.

| | | Liver weight in % weight of animal | Grams % dry weight fatty acids in liver |
|---|---|---|---|
| Pregnant females | *Trygon violacea* ... ... ... | 12·42 | 92·95 |
| | *Torpedo ocellata* ... ... ... | 5·29 | 83·43 |
| | *Scyllium canicula*... ... ... | 7·70 | 99·86 |
| Males | *Trygon violacea* ... ... ... | 8·27 | 85·23 |
| | *Torpedo ocellata* ... ... ... | 5·26 | 76·49 |
| | *Scyllium canicula*... ... ... | 7·97 | 90·58 |

No explanation for this was suggested by Polimanti. A subsequent paper by Reach & Vidakovich went further into the matter, as far as the *Torpedo* was concerned, and Table 172 summarises the results they obtained. Their first set of data, taken from fishes caught in April, gives the figures for just after fertilisation, when the large-

yolked undeveloped eggs fill up the uterine cavity; their second set shows what has happened by the time the embryos have reached the length of about 4 cm., a dry weight of 4·45 per cent. and a nitrogen content of 1·95 per cent. Finally, their third set of data concerns the fish after the embryos have left the uterus. The first point of

Table 172.

Reach & Vidakovich's figures (in grams):

| | Liver | | Ovaries | | Eggs | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Description of animal | Weight in % of weight of animal | Fatty acids % of liver-weight | Weight in % of weight of animal | Fatty acids % of ovary-weight | Weight per fish | Weight each | Fatty acids gm. per fish | Gm. % per fish | Fatty acids gm. per egg | Gm. % per egg |
| "Spring-torpedo" | 4·5 | 44·75 | 0·29 | 1·59 | 63·2 | 7·02 | 5·62 or 6·5 (?) | 0·66 | 0·661 | 9·41 |
| "Summer-torpedo" | 3·14 | 23·6 | 0·23 | 0·13 | 71·1 | 6·95 | 5·08 | 0·66 | 0·499 | 7·18 |
| "Autumn-torpedo" | 4·12 | 22·6 | 1·75 | 5·2 | Embryos born | | | | | |

| | Embryos | | | | | Yolks | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Description of animal | Weight per fish | Weight each | Fatty acid per fish | Fatty acid per embryo | % per embryo | Weight per fish | Weight each | Fatty acid per fish | Fatty acid per yolk | % per yolk |
| "Spring-torpedo" | Not large enough to measure | | | | | — | — | — | — | — |
| "Summer-torpedo" | 9·2 | 0·95 | 0·067 | 0·009 | 0·94 | 61·94 | 6·0 | 5·02 | 0·49 | 8·2 |

Note. In the "Spring-torpedo" the eggs have just come into the uterus; the analyses were made in June, fertilisation probably having taken place in April. In the "Summer-torpedo" embryonic growth has proceeded considerably, the analyses being made in July. The "Autumn-torpedo" has just given birth to the 6–10 embryos and its ovary contains yolked eggs from 10–20 mm. diameter; the analyses were made in August.

interest to be drawn from the table is that the fatty acids decrease notably from 661 to 499 mgm. per egg—a loss of 24·5 per cent. There is no means of telling, of course, whether this loss is all due to combustion, but, even if it were, the amount must be much less than the corresponding loss in a terrestrial egg such as that of the chick, for the latter has 30 per cent. of its yolk as fat and the former only 9·4 per cent. It may therefore be supposed that the main source of energy in the selachian egg is protein. As the table shows, the percentage of fatty acids in the whole egg diminishes from 9·4 to 7·2. As regards the maternal liver and ovaries, Reach & Vidakovich did not confirm Polimanti and Lo Bianco, for the livers of their fishes hardly changed at all in per cent. of the total body-weight, and what change there was during "pregnancy" was in the reverse direction. Nor did the percentage of fatty acids in the liver show any of the changes suggested by Polimanti. Reach & Vidakovich's iodine number results were interesting.

Table 173.

| | Iodine no. | | | | | | Saponification no. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Liver | Ovary | Muscle | Yolk | Embryo (without liver) | Embryonic liver | Liver | Ovary | Yolk |
| "Spring-torpedo" | 120 | 44 | 47 | 129 | — | — | 195 | — | 186 |
| "Summer-torpedo" | 122 | 42 | — | 140 | 38 | 65 | — | — | — |
| "Autumn-torpedo" | 101 | 153 | 43 | — | — | — | 193 | 153 | — |

From this it appears that the fat of the embryonic body has a low iodine value, about the same as that of the adult muscles, but that its liver fat has one twice as high. The yolk fat is twice as unsaturated as that of the avian egg-yolk. Its presence in the ovaries of the "Autumn-torpedo" accounts for the high value found then.

Another selachian, *Centrina vulpecula*, was studied by Kollmann, van Gaver & Timon-David who obtained the following constants from a specimen which seemed to contain in its abdomen practically nothing but eggs and liver:

| | Liver oil | Egg oil |
|---|---|---|
| Density at 15° ... ... ... | 0·9002 | 0·9106 |
| Refractive index ... ... | 1·4689 | 1·4744 |
| Iodine value ... ... ... | 73·4 | 113·9 |
| Saponification value ... ... | 132·3 | 133·7 |

This agrees well enough with the results of Reach & Vidakovich in that the egg oil is particularly unsaturated, cf. the state of affairs in the hen's egg, and the value of ready-made double bonds to the embryo. Apparently the fatty acids of the yolk in this fish have as long chains as those of the liver.

Similar work was done on *Centrophorus granulosus* by André & Canal, who obtained the following figures:

| | Crude oil in % of wet weight | Composition of the crude oil %: Fatty acids (principally clupanodonic) | Squalene | Cholesterol |
|---|---|---|---|---|
| Egg ... ... ... ... ... | 29 | 45·5 | 55·1 | 4·7 |
| Foetal liver ... ... ... | 56 | 32·0 | 66·0 | 3·8 |
| Liver of immature female | 78 | 15·8 | 84·0 | 1·6 |
| Liver of adult female ... | 85 | 8·2 | 91·0 | 1·0 |

They naturally concluded that during ontogeny there was a passage from clupanodonic glycerides and cholesterol to squalene, a passage which was traversed in the opposite direction during the preparation of the eggs by the adult female. Squalene seems to play an important part in selachian metabolism (see p. 350).

## 11·5. Fat Metabolism of Teleostean Eggs

In the teleostean egg all is different. Tangl, in the course of his series of researches on the energy sources of eggs, had accustomed himself to regard fatty acids as the most usual material of this kind, and he received an unexpected surprise when, with Farkas, he investigated the egg of the trout. Estimating the fatty acid content before fertilisation and at hatching, they noted an unmistakable increase, as follows:

| | Amounts in milligrams per egg | | Change | | |
|---|---|---|---|---|---|
| | Before development | After development | Absolute | % Dry weight | % of initial value |
| Wet weight ... ... ... | 88·2 | 83·5 | −4·7 | — | — |
| Water ... ... ... ... | 58·5 | 54·2 | −4·3 | — | — |
| Dry weight ... ... ... | 29·9 | 29·1 | −0·8 | — | — |
| Fatty acids: | | | | | |
| Liebermann's direct saponification method ... ... | 6·4 | 6·64 | +0·24 | +0·825 | +3·75 |
| Ether extraction (probably unreliable) ... ... | 2·82 | 3·55 | +0·73 | +2·51 | +27·6 |

This was confirmed on the eggs of another trout, *Savelinus fontinalis*, by McClendon in 1915, who obtained very similar results, indicating a synthesis of fatty acids during development. These were his results:

| | Amounts in milligrams per egg | | Change % | | |
|---|---|---|---|---|---|
| | Before development | After development | Absolute | Dry weight | Initial |
| Wet weight ... ... ... | 62·0 | — | — | — | — |
| Dry weight ... ... ... | 17·0 | — | — | — | — |
| Fatty acids (direct) ... ... | — | — | — | +0·99 | +5·57 |
| Ether extract ... ... ... | — | — | — | +1·3 | +5·55 |

This curious process appeared again in the work of Dakin & Dakin on the egg of the plaice (*Pleuronectes platessa*):

| | Values in milligrams absolute per 100 eggs | |
|---|---|---|
| | before development | at hatching |
| Wet weight ... ... | 336·0 | 331·9 |
| Dry weight ... ... | 23·9 | 20·8 |
| Fatty acids ... ... | 0·28 | 1·02 |

From these figures it appeared that there was an increase of 0·74 mgm. per 100 eggs, or 264 per cent. of the initial store of fatty acids. Hayes, again, working on the eggs of the lumpsucker (*Cyclopterus lumpus*) found

a rise in total fat from 4·75 to 5·25 per cent. of the wet weight during the 40 days before hatching. And whereas one egg of the Atlantic salmon (*Salmo salar*) contained 6 mgm. fat at the beginning of development it contained 7 mgm. at hatching (65 days).

## 11·6. Fat Metabolism of Mollusc, Worm, and Echinoderm Eggs

The synthesis of fatty acids occurs again in the metabolism of the snail egg (*Limnaea stagnalis*), and it was here indeed that it was first recognised. For in 1853 F. W. Burdach estimated the fat-content of the eggs of the snail from fertilisation to hatching, and the results were published in his thesis *De Commutatione Substantiarum Proteinacearum in Adipem* at Königsberg in that year. His technique was, of course, that in general use at the time, and therefore probably very inaccurate, but the figures were as follows:

| | | Before development | After development |
|---|---|---|---|
| Absolute weight in milligrams per egg | Dry weight ... | 335·5 | 218·0 |
| | Neutral fat ... | 2·25 | 3·5 |
| Fat % of dry weight ... ... ... | ... ... ... | 0·66 | 1·86 |

On the other hand, Fauré-Fremiet, in his comprehensive study of the *Ascaris* egg, observed a diminution of the fraction which he called "fat", but which was really a mixture of neutral fats and ascarylic acid. This fraction fell from 26 per cent. of the dry weight of the egg at the beginning of development to 22·5 per cent. at the end, i.e. a loss of 3·5 per cent. of the dry weight. Then the work of Ephrussi & Rapkine on the developing egg of the sea-urchin, *Paracentrotus lividus*, showed that there was a diminution in the total fatty acids, thus:

| | Hours from fertilisation | | |
|---|---|---|---|
| Total fatty acids | 0 | 12 (gastrula) | 40 (pluteus) |
| % dry weight ... | 21·2 | 19·55 | 17·4 |
| % wet weight ... | 4·81 | 4·43 | 3·69 |

And Payne has shown that fertilisation does not affect the iodine value of the fatty acids in the egg of *Arbacia*.

## 11·7. Fat Metabolism of Insect Eggs

The silkworm egg definitely shows a negative balance of total fatty acids. Tichomirov in 1885 obtained the following figures:

| | % wet weight | | % dry weight | | Change % | |
|---|---|---|---|---|---|---|
| | Before development | After development | Before development | After development | Dry weight | Initial |
| Wet weight ... ... | 100·0 | 88·84 | — | — | — | 11·16 (wet) |
| Dry weight ... ... | 35·51 | 30·20 | — | — | — | 14·9 „ |
| Total ether extract ... | 9·52 | 6·46 | 26·8 | 21·4 | 5·4 | 20·1 (dry) |
| Neutral fat fatty acids... | 8·08 | 4·37 | 22·7 | 12·2 | 10·5 | 46·1 „ |
| Phosphatide fatty acids | 1·04 | 1·74 | — | — | — | — |
| Cholesterol ... ... | 0·40 | 0·35 | — | — | — | — |

Thus 20 per cent. of the initial amount of ether extract in the silkworm egg disappears between the end of the diapause and the time of hatching, and when the neutral fat fatty acids are alone considered the fall reaches 46 per cent. of the initial value. This was later confirmed by Farkas who found a fall of 49 per cent. The only other data which we possess for the silkworm are those of Vaney & Conte, but it is likely that their methods were inadequate and they isolated less than half the amount of total fatty acid found by Tichomirov. Their figures were as follows:

| | Fatty acids in % dry weight of egg |
|---|---|
| 4 days after laying ... ... ... ... | 7·21 |
| 6 days after laying ... ... ... ... | 6·68 |
| End of the diapause (9 months after laying) ... | 5·35 |
| Hatching (10 months) ... ... ... ... | 4·88 |

and suggested a loss of 2·33 per cent. of the dry weight, or 32·3 per cent. of the initial weight. Kaneko has made a histochemical study of the fat in the silkworm egg, especially concerning himself with its localisation. Another piece of information concerning fat metabolism in insect eggs is due to Weinland, who made a "*Brei*" of the eggs of the blowfly, *Calliphora vomitoria*, and adding Witte's peptone, found after some days of anaerobic action a decrease of about 30 per cent. in total fatty acids. This stood in the sharpest contrast to the behaviour of "*Breis*" of larvae and of pupae, which have a remarkable power of converting peptone and even protein into fatty acids. It would thus appear that *Calliphora vomitoria* embryos consume a large amount of fat during their development, just as do those of *Bombyx mori*. Then for the grasshopper, *Melanoplus differentialis*, we have the careful work of Slifer, who found 0·352 mgm. total fatty acid at the beginning of development and 0·14 mgm. at the end; each egg, therefore, lost 54·3 per cent. of its initial store. This fits in well with the respiratory quotients of 0·7 to 0·8 which, according to Bodine, are always found for this material.

An interesting study of the fat metabolism of the eggs of the lackey moth or tent caterpillar, *Malacosoma americana*, was made by Rudolfs. Fig. 369, constructed from his data, shows the progressive decline in total fatty acids during the development of this insect. Evidently a very large proportion is used up, probably by combustion, in agreement with the findings on the silkworm and the grasshopper. As the chorionic coverings constitute 52 per cent. of the whole egg-mass, the fat-content of the egg itself on laying would be about 9·0 per cent. dry weight. As the water-content decreased only slightly during development (see Fig. 369), the fat/water ratio decreased a good deal; thus at laying there would be 9 mgm. of fatty acids per 100 mgm. of water, but at hatching only about 2 mgm.

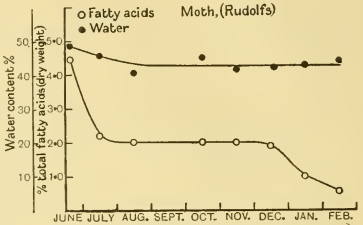


Fig. 369.

## 11·8. Combustion and Synthesis of Fatty Acids in Relation to Metabolic Water

It is exceedingly interesting to note the similarity between the insect egg and the egg of the chick. All these terrestrial eggs burn relatively large amounts of fat, and, though this is perhaps associated with the ease with which fatty acids can be stored in a small space, yet it may also be related to the fact that fat, when it burns, leaves behind all its original weight in the form of water. We have already seen that one of the most difficult problems confronting the earliest terrestrial animals must have been the proper supply of water for their embryos, and it is therefore likely that the use of fat as the principal source of energy is associated with the importance of the water balance. Aquatic embryos which have no trouble in this direction do not, as far as we know, burn large quantities of fat. Babcock and others have calculated that 100 gm. of fat on combustion yield 107·1 gm. of water, 100 gm. of carbohydrate, 55·5 gm. of water, and 100 gm. of protein only 41·3 gm. of water. From Table 126 of Section 7 it is clear that the chick burns much more fat than the frog (approximately 80 per cent. in the former case and 20 per cent. in the latter case, of the total material combusted), and

this may be regarded as a consequence of their special needs. Energy is no doubt the same from whatever source it comes, but one source may be more convenient than another. In the hen's egg, according to Murray's data, 6·32 gm. of water are lost from the system during its development, and 2·01 gm. of solid. As 91 per cent. of the solid lost is fat, about 2·1 gm. of "metabolic water" are added to the egg, so that 8·4 gm. of water would have been lost if fat had not been burnt, or 40 per cent. more than what actually is lost. The chick in its closed terrestrial box cannot afford to despise this extra two grams of metabolic water.

Table 174.

| Animal | Aquatic or Terrestrial embryo | Fatty acids combusted during development in % of the total fatty acids present in the egg at the beginning. (All figures for the whole of development) | Investigator |
|---|---|---|---|
| Chick ... ... | T. | 60 | Murray; Idzumi; Sakuragi, etc., etc. |
| Silkworm ... ... | T. | 48 | Tichomirov; Vaney & Conte; Farkas |
| Lackey moth ... | T. | 85 | Rudolfs |
| Grasshopper ... | T. | 54 | Slifer |
| Marine turtle ... | A. (?) | 34 | Karashima |
| Frog... ... ... | A. | 28 | Parnas & Krasinska; Barthélemy & Bonnet, etc. |
| Torpedo ... ... | A. | 24·5 | Reach & Vidakovich |
| Plaice ... ... | A. | <10 | Dakin & Dakin |
| Sea-urchin ... ... | A. | 20 | Ephrussi & Rapkine |

It is difficult to ascertain exactly how much fat is combusted by different animals; for as it burns away, leaving no measurable incombustible residues, its combustion-rate cannot be calculated from them, and no one can say *a priori* what proportion of the carbon dioxide eliminated comes from fatty acids. Then in some animals, as we have seen, there is a veritable synthesis of fatty acids during embryonic development, obscuring any combustion of them. One could therefore predict that the difference between terrestrial and aquatic embryos would not be so clear-cut in the case of fat combustion as it is in the case of protein combustion. However, Table 174, constructed in the same way as Table 162 in Section 9·15, shows that embryos do divide into the two classes according to their environment in this case also. Terrestrial eggs burn between 60 and 70 per cent. of their initial fat stores, and aquatic ones only about 20 per cent. The difference is really even more striking than it seems

from the table, for terrestrial eggs store the greatest amount of fat, so that 20 per cent. of the fat in the hen's egg is a good deal more proportionately to the embryonic weight than 20 per cent. of the fat in the sea-urchin's egg. But any direct comparison between the terrestrial or aquatic environment and the amount of fat stored in the egg is not possible, for, as Table 31 (in which the fat/protein ratios of many eggs are given) shows, there is no strict correlation. The terrestrial sauropsid egg contains much more fat than any of the others, relatively to the protein, but the insect egg, which is also terrestrial, contains very little (e.g. silkworm and lackey moth). In other words, the correlation between environment and material used as energy source does not appear until one knows the exact amount of material combusted during development, and expresses that in terms either of the amount of it originally present, or of the total material combusted.

Now we have seen that, in six separate instances, an absolute increase in fatty acids has been observed during development, one of the animals in question being a urodele amphibian, two being teleostean fishes, and one a pulmonate gastropod (all aquatic embryos). It is likely, in view of the discussion on p. 350 about the oil drops in fish eggs, etc., that this list would be much prolonged if more investigations were made of their chemical embryology.

What is the nature of this fat synthesis? Tangl & Farkas, who were the first in recent times to establish it experimentally, thought that the trout egg contained "glycoproteins" which were broken down to carbon dioxide and water, but which also went to form glycogen and fat. They believed, in fact, in a predominance of protein metabolism and in a formation of fat from protein, the waste nitrogen being retained in the egg in the form of urea, and liberated at hatching to the exterior. Tangl & Farkas, however, made no attempt to demonstrate the presence of urea. Then Gortner, in his studies on the protein metabolism of the trout and salamander egg, concluded that this could not be the case, as no urea or uric acid was to be found in the eggs at the time of hatching. Unfortunately, he neither identified the urea and uric acid specifically nor brought forward evidence in disproof of their presence by the use of appropriate tests. In view of the importance of the subject, it would be highly desirable for a new investigation to be made of the protein metabolism of the trout egg. The fact of the matter is that neither

Tangl & Farkas' hypothesis nor Gortner's experiments throw any light on the origin of the synthesised fat in these eggs. Apparently the only reason why Tangl & Farkas suggested a glucoprotein as the energy source was because they could not imagine fat being synthesised from anything else. Each egg, they found (by analysis), expended 6·68 cal. during development, and they reasoned that to produce this energy from 518 eggs (their experimental number) 1·67 gm. of glucoprotein (9·7 Cal.) must be broken down to 0·38 gm. of fat (3·5 Cal.) and 0·30 gm. of glycogen (1·3 Cal.), and all of the nitrogen retained in the form of urea (0·57 gm., i.e. 1·40 Cal.), the difference between these heat values being carbon dioxide and water with a heat value of 3·5 Cal. As they found experimentally a heat loss of 3·46 Cal., they considered that their theory covered the facts sufficiently well, but it would be easy to think of several others equally convincing. They found, moreover, that the loss of carbon, 46·3 per cent., did not agree with the expected loss, 68 per cent., and they suggested that perhaps it was not all eliminated as carbon dioxide, but retained in some other form.

The problem is undoubtedly a difficult one and at present there is no solution for it, but there are two points which seem to have been overlooked by all those who have so far considered it. In the first place, even when the increase in "fat" has been demonstrated to be an increase of fatty acids, and not of total ether extract, it has always been assumed that no breakdown of these can have been occurring. Yet it is possible that a catabolism of fatty acids might exist masked by a reverse process, so that, as the fats were destroyed, more were formed from some other source so as to over-compensate for the fat combustion. The second suggestion is that substances such as spinacene (see p. 350), which have recently been found in fish eggs, may play a very important part as energy sources. So far there is no experimental foundation for this idea, but it is quite conceivable that spinacene or squalene might be oxidised directly for energy, or that such substances might be the origins of the synthesised fat in certain aquatic eggs. It is indeed difficult to see any biological reason why this fat synthesis should go on, and it is noticeable that it never reaches great dimensions—8 per cent. in the salamander, 55 per cent. in the snail (if we may trust Burdach's figures), and 5 per cent. in the trout. It is true that the increase in the plaice is 264 per cent., but the total quantities in question there are exceedingly small relative to

the total amount of substance in the eggs. It will be admitted that the whole subject of fat synthesis in these aquatic eggs urgently needs re-examination.

## 11·9. Fat Metabolism of Mammalian Embryos

It has often been asked how fatty acids arise in the mammalian embryo, since there is no yolk from which they can be transferred. The obvious answer is that the foetal fat is supplied through the placenta, but this has to meet the objection that there is a good deal of evidence against the existence of this transportation. It would perhaps be more suitable to delay the consideration of this question till the Section on the placental barrier, but it is too intimately associated with fat metabolism. That the fat-content of the foetal blood is independent of the fat-content of the maternal blood was suggested by the experiments of Oshima, who in 1907 studied the ultra-microscopic particles of the foetal blood. Neumann had shown some years before that the concentration of these in blood ran closely parallel to the fat-content as determined chemically, and they are probably identical with the "chylomicrons" of Gage & Fish. Oshima found that in the guinea-pig the maternal blood has always a "mässiger," "zahlreich", or "massenhaft" number of fat-particles, but the early foetal blood "spärlich". As the embryo developed, however, the number of fat-particles in its blood rose greatly, and at birth nearly, if not quite, equalled that in the maternal blood. It was significant that the condition of the foetal blood seemed to be independent of the mother, for fasting, Oshima found, would easily reduce the fat-particles in the mother's blood to few or none, without having any effect on the foetus, which was always as rich in fat-particles as it was scheduled to be at the time of development in question. On the other hand, if fat was fed to the mother in large amounts there was no effect on the embryonic blood, although a large one on the maternal blood. Oshima naturally concluded that fat was not transported from the maternal to the foetal circulation.

This received later a quantitative backing from various investigators. Ahlfeld very early had reported large and inconstant differences between maternal and foetal blood-fat in the dog, and had shown that bacon feeding affected the maternal circulation only. Kreidl & Donath in 1910 estimated the fat-content of guinea-pig foetal blood in normal conditions (development time not stated) at

746 mgm. per cent. as against 314 mgm. per cent. in maternal blood, in both cases nearly all in the plasma. They thought it likely that a synthesis of fat by the placenta must occur, but they could not demonstrate the presence of any lipolytic enzyme in it. The other attempts which have been made to gain knowledge about the lipolytic enzymes of the placenta will be discussed in the section on that organ. More recently Slemons & Stander obtained the following figures for foetal and maternal blood at term in man:

| | | Average milligrams % |
|---|---|---|
| Whole blood | Maternal ... | 908 |
| | Foetal ... | 707 |
| Plasma | Maternal ... | 942 |
| | Foetal ... | 737 |

and concluded that the fatty acids of the embryo were not directly derived from the mother. Murlin & Bailey found the same relation in man at term, though their figures were lower than those of Slemons & Stander.

| | | Average milligrams % |
|---|---|---|
| Whole blood | Maternal ... | 550 |
| | Foetal ... | 266 |

Murlin & Bailey believed that an association existed between fat-content of foetal blood and severity of labour.

Another line of evidence against the passage of fatty acids from maternal organism to embryo arises from the fact that, when fats are earmarked in one way or another, they do not subsequently appear in the fat depots of the foetal tissues. Two principal methods have been used for this purpose, firstly, the marking of fatty acids by stains and dyes, and, secondly, the use of special or highly unsaturated fats which can easily be recognised at the other end. Hofbauer was perhaps the first to utilise these methods, for, in his many-sided study of the metabolism of the placenta in 1905, he fed fats stained in various ways to pregnant animals, and stated that he observed traces of the colour afterwards in the foetal fat. The colour can only have been in traces, for in the hands of subsequent experimentalists the results of this type of experiment have been uniformly negative. Thus Gage & Gage in 1909 fed fat stained with Sudan III to various kinds of pregnant animals, and never observed the slightest indication of a passage through the placenta. The maternal fat depots would be brilliantly pink or red, while those of the embryo would be perfectly

colourless. Similar experiments were carried out by Mendel & Daniels in 1912, using Sudan III and Biebrich scarlet, and working on pregnant rats and cats, but always the embryos remained absolutely colourless, although the maternal fat was brightly coloured. Baumann & Holly later still obtained the same quite negative results. There is some likelihood that the placenta could detach the dye from the fatty acids.

Then Hofbauer in 1905 fed cocoa butter to dogs, and claimed to have identified lauric acid in the foetal fat after some days, but its presence was not really established, and the experiments might well be repeated. Thiemich's work was better, although it antedated that of Hofbauer by some years. Thiemich fed certain dogs on cocoa butter and others on linseed oil cake, with the following results:

| | Iodine value of fat in food | Iodine value of foetal fat |
|---|---|---|
| Cocoa butter ... ... | 8 | 71·3–73·1 |
| Linseed oil ... ... | 120 | 69·3–70·3 |

from which he naturally concluded that the foetal fat maintained its accustomed iodine number quite unchanged, no matter what was the degree of unsaturation of the fatty acids entering the maternal body. Thiemich did not estimate the iodine value of the maternal fat, but assumed that it would vary closely with the food fed. In a later paper, however, he modified his conclusions somewhat, because he estimated simultaneously the iodine value of the maternal fat, and found that it did not change as much as he had expected, e.g. only from 30 to 50. Becker afterwards discussed the question further without adding anything to it. Wesson in 1926 using the method of bromine addition compounds, fed cod-liver oil to one set of pregnant rats, and ordinary butter to another set, and found, although the bromine number of the fed fats was extremely different, hardly any difference was to be seen in the foetal fat.

| | | Bromine number of body fat |
|---|---|---|
| Cod-liver oil (bromine number 0·210) | Foetal ... | 0·021 |
| | Maternal ... | 0·042 |
| Butter (bromine number 0·0031) | Foetal ... | 0·025 |
| | Maternal ... | 0·011 |

This is probably the most reliable, as it is the most recent, work on this subject. Wesson suggested a choice between three possible explanations: (1) that the placenta has a selective action, rejecting all

fatty acids except those of a certain degree of saturation, (2) that the embryo can alter fats arriving at it by hydrogenation or dehydrogenation, or (3) that fatty acids do not pass the placenta at all, and that the embryo forms its supplies from carbohydrate, or possibly from protein. In spite of the difficulties inherent in the third possibility, it was the one which Wesson himself considered most likely. For the further discussion of this problem, see the Section on placental permeability.

As regards the fat in the embryonic body of the mammal, Derman has stated, as a result of the employment of histochemical methods, more or less specific, that, up to the first month, the fat depots of the human embryo contain mostly neutral fat and cholesterol esters, less phosphatides and almost no free fatty acids, but, in the later stages, neutral fat and cholesterol esters only. For other histochemical work see Froboese and Berberich & Bär. Raudnitz found that the melting-point of human fat was higher the younger the body, thus:

| | °C. |
|---|---|
| 8·5 month foetus ... ... | 47·2 |
| 2 days after birth ... ... | 43·8 |
| 1 year ... ... ... ... | 30·2 |
| 26 years ... ... ... ... | 27·0 |

but no explanation is available for this phenomenon. Another curious observation, due to Dobatovkin, is that as growth proceeds the percentage of fatty acids liquid at room temperature increases. Thus, of human fat from the shoulder region:

| | Liquid % | Solid % |
|---|---|---|
| At birth ... ... | 52·7 | 39·6 |
| 6 years old ... ... | 82·2 | 10·7 |

The same fact, expressed more scientifically, is to be found in the data of Egg and of Jaeckele & Knopfelmacher, the latter of whom estimated the percentages of the three most important fatty acids as follows:

| | At birth % | Adult % |
|---|---|---|
| Oleic ... ... | 65·04 | 86·21 |
| Palmitic ... ... | 27·81 | 7·83 |
| Stearic ... ... | 3·15 | 1·93 |

It is striking, from a comparative point of view, that the fat of the egg-yolk of the chick should contain only 5 or 6 per cent. of oleic acid. The data of Jaeckele & Knopfelmacher do not extend back

into pre-natal life, but it may be presumed that the process is continuous.

An interesting investigation which involved several aspects of embryonic fat metabolism was made by Imrie & Graham, who estimated the amount of fat and its iodine value in the embryonic and maternal livers of guinea-pigs during gestation, using Leathes' method. Fatty infiltration of the liver in the non-pregnant animal is known to be due to the entry of tissue fat into the liver, for the iodine value characterises the fat normally present in the liver and in the other tissues, being high in the former and low in the latter. The question which Imrie & Graham set out to solve was whether the embryonic liver behaved in the same way, or whether it evinced any special physiological characteristics in its reactions to disturbances of fat metabolism. "It seemed reasonable to suppose", they said, "that the embryonic tissue might possess an avidity for food material such as fat and that this might evidence itself by a greater accumulation of fat in the embryonic than in the maternal liver, if the fat were mobilised experimentally." Obviously it was first necessary to establish normal curves for the embryonic liver fat, and this in itself brought some interesting results, as may be seen from the curves plotted in Fig. 370. It was found that during development the guinea-pig embryo accumulates considerable stores of fatty acids in its liver. At first (up to body-weight 30 gm.) the percentage of fatty acids in the embryonic liver does not differ much from that in the maternal liver, but after that point the percentage in the former rises sharply to reach a maximum at about 80 gm. body-weight (roughly 65 days conception age), after which there is no further change until birth. Immediately upon birth, however, the fatty acid percentage drops

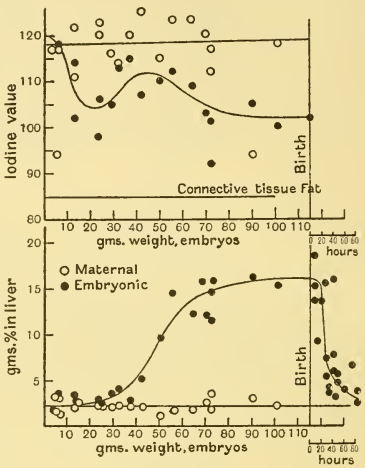



Fig. 370.

rapidly, and after 80 hours of post-natal life has reached a level very like that of the maternal liver. As the figure shows, the fatty acid concentration in the maternal liver is unaffected by gestation. "The livers obtained from young embryos", said Imrie & Graham, "resembled histologically and chemically adult hepatic tissue, whereas in embryos fully developed the liver was yellow in colour and showed chemically a large accumulation of fat. . . . A fatty infiltration of a physiological kind occurs in the livers of embryonic guinea-pigs." The synchronous behaviour of the iodine value was very interesting, for, as Fig. 370 shows, it remained throughout the process in the neighbourhood of 110, i.e. much above the iodine value of the embryonic connective tissue fat, which had an iodine value of about 85. The rises and falls on the curve are probably not significant, but the fat of the embryonic liver seems always to be a little less unsaturated than that of the maternal liver. The fact, however, that its iodine value was 110 precluded all possibility of the infiltration being one of tissue fat, as in the adult. It seems likely also that there is a slight decline in the iodine value of the embryonic liver fat; for embryos under 40 gm. it could be said to be generally 111, and above 40 gm. nearer 103 or 104. The difference between the average maximum and minimum fat values of the embryonic liver is 9·94 per cent. If we assume that this was fat coming from the connective tissue and having an iodine value of 85, and added to the 3·16 per cent. having an iodine value of 109, the resulting iodine value should be 91, but instead it is at its lowest 103. It would be very interesting to have a parallel set of data for the liver of the embryonic chick, for, as we have already seen, there are ocular evidences for a fatty infiltration there also.

It is generally admitted that the liver desaturates fatty acids brought to it, and that when it is infiltrated with connective tissue fat it falls behind in this work, with the result that the low iodine value is found. One must therefore conclude either that the liver of the embryonic guinea-pig can desaturate relatively much larger amounts of imported fat than is the case in extra-uterine life, or that the fat taken up by the embryonic liver is fat which has already been desaturated by the maternal liver or by some other tissue. As desaturation is the preliminary to combustion, Imrie & Graham's results raise the suspicion that fatty acids may play a more important part in the provision of energy for the mammalian embryo than has been sup-

posed in the past. Imrie & Graham, however, in view of the sudden and rapid decrease in the liver fat after birth, thought it likely that it was utilised for some special purpose then, which may well be the case.

They next proceeded to mobilise the fatty acids of the body by giving phloridzin and by subjecting the animals to a fast. The results were as shown in Fig. 371. Evidently phloridzin and fasting will produce a fatty infiltration of the embryonic liver. The graph also shows that treatment which will not very greatly affect the maternal liver will have a considerable effect on that of the embryo. From the data on the iodine value it can be seen that, simultaneously with the entry of fresh fat into the embryonic liver, the iodine value of the liver fat falls, just as would be expected if such fat was coming in from the other tissues. Here again the maternal liver is hardly influenced. Experiments similar to the foregoing were carried out on pregnant guinea-pigs with embryos over 40 gm. in weight, and from these it appeared that the physiological infiltration of about 15 gm. per cent. is by no means the maximum capacity of the embryonic liver, for by means of phloridzin the amount was raised to as much as 30·49 per cent. (wet weight). These experiments showed that phloridzin must pass the placenta, and make it highly probable that the physiological fatty infiltration is not derived from the tissue fat but some other source. Perhaps this other source is that postulated by Wesson in his explanation of the increasing fat-content of the embryonic body and the impermeability of the placenta to fatty acids.

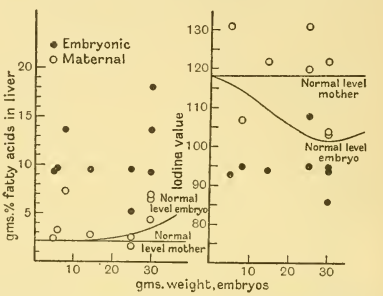



Fig. 371. The normal data are here represented by continuous lines; the phloridzin data by points not joined together.

One of the statements which has entered the literature without much justification is that embryonic cells (and carcinoma cells too) will not show fatty infiltration when treated with phosphorus. This was said to be the case by Hess & Saxl who made *in vitro* experiments on guinea-pig and rabbit tissues (liver and kidney). Some years later

doubt was thrown on this by Schwalbe & Mücke who could not confirm it, using very similar technique, and finally Rosenfeld, who worked with whole animals, found that foetal cells showed just as much fatty infiltration as adult ones.

| | | % fatty acids maternal liver | % dry weight foetal liver |
|---|---|---|---|
| Dog | Normal ... ... | 28·5 | 2·91 |
| | Phosphorus poisoning ... | 40·0 | 10·06 |

Whether carcinoma cells would also show fatty infiltration he did not try, but as he once obtained 2·3 per cent. of fat from a liver metastasis in man, he was of the opinion that they would.

## SECTION 12

# THE METABOLISM OF LIPOIDS, STEROLS, CYCLOSES, PHOSPHORUS AND SULPHUR

### 12·1. Phosphorus Metabolism of the Avian Egg

The study of the distribution of phosphorus in a living system is important for it exists in so many types of compound—lipoids, phosphoproteins, nucleoproteins, hexosephosphates, etc.—that a remarkable survey of wide tracts of the mechanism of the egg can be obtained by simply observing where the phosphorus is. It is for this reason that the paper of Plimmer & Scott in 1909 may be called classical, for it threw more light on the complex transformations going on during the incubation of the hen's egg than any other single paper before or since. It is true that they were not the first to study the behaviour of the phosphorus fractions during development, for already in 1877 Hoppe-Seyler had estimated the phosphorus in the yolk, and had calculated thence the lecithin. He deserves much credit as being the first to approach these problems from the quantitative point of view, but his results are of little more than historical interest. Then in 1893 Maxwell went into the subject again. He seems to have been in the grip of a preconceived theory associated with work on plants, and he certainly had the misfortune to use untrustworthy methods, but he succeeded in showing that lipoid phosphorus preponderated at the beginning of development, and non-lipoid phosphorus at the end.

| | % of the total phosphorus | |
|---|---|---|
| | Ether-soluble | Not ether-soluble |
| Beginning ... ... | 58·5 | 41·5 |
| End ... ... | 27·0 | 73·0 |

The rest of Maxwell's figures compressed great variations into a minimum of data, and did not invite a belief in their reliability. Worse still, he counted the phosphorus in the vitellin as "mineral" phosphorus. In 1908 a much better piece of work was done by Carpiaux, who observed an increase of inorganic phosphate during development at the expense of the ether-soluble phosphorus. He defined

"inorganic" phosphorus as all the non-lipoid phosphorus, and the figures which he obtained were as follows:

| Days | Milligrams per egg non-lipoid phosphorus | Milligrams per egg lipoid phosphorus |
|---|---|---|
| 0 | 75 | 150 |
| 6 | 128 | 146 |
| 7 | 127 | 135 |
| 14 | 137 | 108 |
| 18 | 182 | 50 |

About the same time Mesernitzki published in a short paper the results of some experiments in which he had followed the amount of ether-soluble phosphorus in the whole egg during incubation. His data, arranged graphically and expressed as lecithin in grams per 100 gm. dry weight of egg-contents, are to be found in Fig. 372.

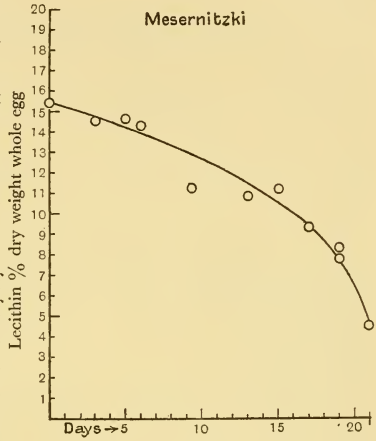


Fig. 372.

Before going on to discuss Plimmer & Scott's data for the phosphorus distribution in the egg, we must touch on the question whether the contents of the egg receive any accession of phosphorus from the shell in the case of birds, as has from time to time been supposed. In the analyses of Voit, Hermann, Forster, Feder & Stumpf, the yolk contained 203·86 mgm. of phosphorus at the beginning of development, while the white contained 7·04 mgm., a total of 210·9. At the end of development the egg-contents contained 237·5 mgm., i.e. an increase of roughly 26 mgm., but Voit and his assistants did not mention this fact in their conclusions, and probably, if they noticed it, put it down to experimental error. In Pott & Preyer's experiments of 1882, the following figures were obtained:

| | $PO_4$ mgm. |
|---|---|
| In shell at beginning ... ... ... | 44 |
| In egg-contents at beginning ... ... | 228 |
| In shell at end ... ... ... ... | 42 |
| In egg-contents at end ... ... ... | 224 |

They concluded that the differences were within their experimental error, and that the shell did not provide any phosphorus for the egg-interior.

Carpiaux in 1908 made a certain number of shell-analyses with the following results:

| | Carpiaux % dry weight | | Burke, Pinckney & Jones % dry weight | |
|---|---|---|---|---|
| | Unincubated | Incubated | Unincubated | Incubated |
| Calcium oxide ... ... | 54·30 | 54·86 | 52·69 | 52·77 |
| Phosphorus pentoxide ... | 0·31 | 0·32 | 0·371 | 0·403 |

The ratio calcium/phosphorus was therefore in the former case 175·1/1·0, and in the latter case 174·1/1·0. The amount of phosphorus taken from the shell must then be very minute, for according to other data of Carpiaux the shell loses 162·2 mgm. of calcium oxide to the egg-interior during the 21 days, and, as the calcium/phosphorus ratio in the shell is almost identical before and after, the phosphorus lost, if any, must bear the same ratio to the calcium lost, i.e. 175 to 1, which would work out at not more than 0·9 mgm. of phosphorus pentoxide. Finally Harcourt & Fulmer in 1908, Delezenne & Fourneau in 1918, and Burke, Pinckney & Jones in 1925 showed absolutely no increase in total phosphorus of the egg-interior during the 21 days of development.

Plimmer & Scott made use of the following classification of phosphorus fractions:

(1) Inorganic phosphorus soluble in water and acids[1].

(2) Organic phosphorus compounds, soluble in water and acids, i.e. guanylic acid, inosinic acid, phosphocarnic acid, free glycerophosphoric acid, hexosephosphates and all similar bodies. This fraction would also include pyrophosphate.

(3) Compounds such as lecithin and kephalin soluble in ether.

(4) Nucleoproteins and similar bodies insoluble in water.

(5) Phosphoproteins and similar bodies insoluble in water.

Plimmer & Scott extracted the eggs and embryos with ether, alcohol, 1 per cent. hydrochloric acid, distilled water, etc., and then estimated the inorganic, organic, and total phosphorus by the Plimmer-Bayliss

[1] It should be emphasised that organic phosphorus compounds of a labile character such as creatine-phosphate, which have been discovered since 1909, would appear in this fraction. An investigation of the appearance of these in the chick embryo is urgently needed.

modification of the traditional Neumann method on each fraction. The dry protein residue was split up into nucleoprotein and phosphoprotein by treatment with 1 per cent. sodium hydroxide at 37° for 24 hours, which converts the phosphoproteins, vitellin (and livetin?) into inorganic phosphorus, but does not hydrolyse the nucleoproteins. Then finally adding up the organic and inorganic phosphorus in the different fractions, Plimmer & Scott obtained their balance sheet of the phosphorus transformations. Thus they found 215·56 mgm. phosphorus pentoxide in an average egg as the total, and they recovered in the fractions 211·75 mgm. or 98·3 per cent., a result which gave them confidence in their technique. For the whole of development they found very notable changes, thus:

| | % of the total phosphorus | |
|---|---|---|
| | Beginning | End |
| Inorganic P ... ... ... | Trace | 60·0 |
| Water-soluble organic P ... | 6·2 | 8·6 |
| Ether-soluble organic P ... | 64·8 | 19·3 |
| Vitellin P ... ... ... | 27·1 | 0·0 |
| Nucleoprotein P ... ... | 1·9 | 12·0 |

The broad outline demonstrated, then, that the inorganic phosphorus had risen at the expense of the ether-soluble phosphorus, that the vitellin had also disappeared, and that the nucleoprotein had increased. The picture is better seen, however, on a graph constructed from Plimmer & Scott's data, in Fig. 373. It is composed of two principal change-overs, firstly, the "lecithin" or ether-soluble phosphorus, and the inorganic phosphorus, the former descending from 60 to 20 per cent., and the latter ascending from 0 to 60 per cent.; and secondly, the phosphoprotein and nucleoprotein phosphorus, the former descending from 30 per cent. to nothing and the latter rising from 1 to 15 per cent. During all this, the water-soluble organic phosphorus remains practically at a constant value, except for a dip on the 20th day, which probably has no significance. From the graph, then, it would seem as if the lecithin is transformed mainly into inorganic phosphorus, and the phosphoprotein into nucleoprotein, but we must not overlook the probability that a certain fraction of each precursor is devoted to each end product. Fourteen years after Plimmer & Scott's paper, Masai & Fukutomi undertook an exactly similar research. Their figures are assembled in Fig. 374, from

which it can be seen that they confirmed Plimmer & Scott in every particular, except that they did not distinguish between phosphoprotein and nucleoprotein phosphorus. In both cases the facts agree excellently with the state of affairs long known to morphologists, namely, that the processes of ossification in the embryonic bones are proceeding during the last week of incubation. Obviously the lecithin phosphorus is transformed into phosphorus in the embryonic bones. As can be seen from Fig. 406, the curve for increase of calcium in the embryo runs almost exactly parallel to this curve for increase of inorganic phosphate. The increase in inorganic phosphate, however,

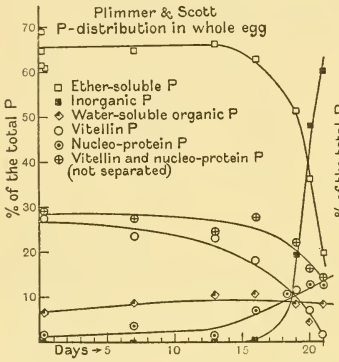



Fig. 373.

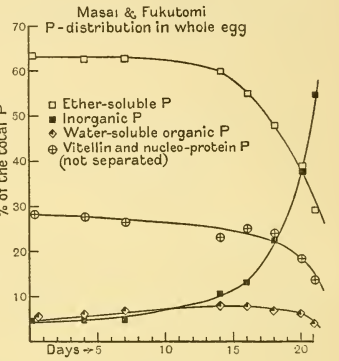



Fig. 374.

is not confined to the embryo as Figs. 375 and 376 show, and this suggests that calcification of the bones is not the only destination for the inorganic phosphorus. The albumen becomes acid (see Fig. 211) towards the end of incubation, and, though from the work of Vladimirov it seems as if the increasing carbon dioxide elimination is not responsible for this, but rather some fixed acid produced by the embryo, yet the albumen never becomes more acid than *p*H 6·1, although its titratable acidity shows a rather greater proportionate increase. A buffer action is, in fact, indicated. If, then, the rise in inorganic phosphate is not confined to the embryo, it is not unreasonable to suppose that sodium or potassium phosphate may be the substances responsible for the buffer action.

Attention may now be directed to the protein phosphorus. As can be seen from both graphs (Plimmer & Scott; Masai & Fukutomi), the increase in nucleoprotein phosphorus does not account for more than a half or two-thirds of the vitellin phosphorus disappearing. This is seen by the decrease which the total residual protein curve exhibits. Some of the vitellin phosphorus is therefore used to make something else besides nuclein phosphorus. It is very interesting to see that the nucleoprotein phosphorus increase is not only in the embryo, but also outside it, a fact due to the separation made by Plimmer & Scott being between the embryo and membranes, so that

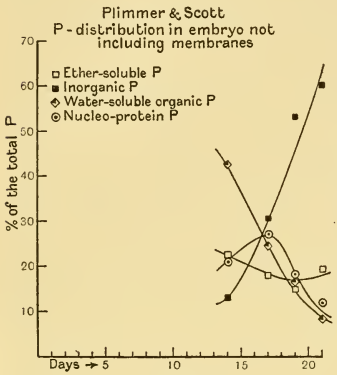



Fig. 375.

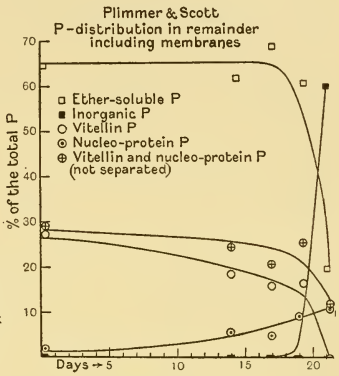



Fig. 376.

the latter were included with the remainder of the egg. This is a demonstration that their content of nuclein is not insignificant. In the embryonic body the percentage of nuclein phosphorus seems to have a peak on the 17th day, but this is probably not real, as the curves are percentage curves and not curves of absolute magnitude. Doubtless the number of milligrams of nuclein phosphorus increase without a break in the embryo, but after the 17th day the enormous increase of inorganic phosphate in the chick's body causes a diminution in the percentage of nucleoprotein phosphorus. It has been suggested that the manufacture of nuclein proceeds externally to the embryo to some extent, and that the nuclein is absorbed afterwards, and, in view of Sendju's results on the purine content of the

yolk, this is probably the case, so that the presence of the membranes in Plimmer & Scott's "remainders" will perhaps not wholly account for the rise in nucleoprotein phosphorus outside the embryo. The ether-soluble phosphorus outside the embryo behaves in a manner very similar to that seen on the curves for the whole egg, but inside the embryo it seems to show a fall towards the end of development. This may possibly be a chemical expression of cephalocaudal differential growth (see p. 583); in other words, the lecithin phosphorus in per cent. of the total phosphorus in the embryo may decline owing to the fact that the brain and central nervous system of the embryo are declining in percentage of the total weight. Plimmer & Scott's figures for the phosphorus distribution in the embryo were not very numerous, and it would be exceedingly desirable to extend them in a backward direction, so that we might know the phosphorus distribution in an embryo 3 or 4 days old. It might be predicted that the ether-soluble phosphorus would be high and everything else low, except, perhaps, the water-soluble organic phosphorus. In this connection the results of Marza are of interest. Using the histochemical method of Romieu, which is said to indicate the presence of lecithin, he observed a distinct lag between the formation of the neural portions of the early chick embryo and the appearance of lecithin. A gradient was also perceptible, the amount of lecithin at a given time decreasing from cephalic to caudal end. This would suggest that the morphological pattern of the early neural elements is sketched out, as it were, in a protein-carbohydrate medium, while the lipoids, so essential a constituent of the fully developed central nervous system, are brought in later. All this fits in remarkably with what we know about the composition of white and yellow yolk (see p. 286). Another point of some interest arises out of Fig. 373, where the vitellin phosphorus of the whole egg seems almost as abundant on the 13th or 14th day of development as it was on the 3rd or 4th. This is another reflection of the fact already so often seen from different angles, namely, that the yolk is "a remoter and more deferred entertainment than the white".

The water-soluble organic phosphorus is perhaps the most interesting of all the fractions. On both the graphs for the phosphorus distribution of the whole egg, it can be seen that this fraction, beginning at about 5 per cent., increases slowly to about 10 per cent. in the middle of development, and finally falls away again to its

initial value, or rather below it. This must surely be due to the fact that, in the water-soluble phosphorus, we have the principal form in which phosphorus is transported from place to place. It would never be likely to make up a great part of the total phosphorus, for its concentration would always be kept low owing to its destruction as soon as it was formed, but yet a slight rise might be expected to hint at a more intense transport of phosphorus. The water-soluble organic phosphorus is perhaps especially associated with ossification, as an intermediate stage in the transformation of ether-soluble into inorganic phosphorus. In this fraction would be included hexosephosphates, glycerophosphates, inositolphosphates (phytin-like bodies), etc. From the point of view of bone formation great interest attaches to these effects. Robison in 1923 discovered an enzyme in calcifying bone which had the power of breaking down hexosemonophosphoric acid to inorganic phosphate, and also attacked the glycerophosphoric esters, but no cyclose phosphorus compounds. It was not present in other tissues besides bone, except to a slight degree in the intestine and kidney (Robison & Soames; Robison & Kay; Robison & Goodwin), and Robison & Martland showed that there was concurrence between the onset of calcification and the appearance of the bone phosphatase. Subsequent work indicated that the bone phosphatase was exclusively concerned with calcification in growth, while the kidney phosphatase was concerned with normal functioning. Kay found that on the 12th day of development in the chick there was three times as much phosphatase in the leg bones as on the 21st day, and that on the 12th day the water-soluble organic substrate was present to a much greater extent than in the unincubated egg. Now in the curves for the embryo only (Fig. 375) it is noticeable that just during the period of most vigorous bone formation, i.e. from the 12th to the 21st day the water-soluble organic phosphorus is steadily decreasing. It is as if the water-soluble organic phosphorus was concentrated to a high level in the embryonic body during the first half of incubation, only to be transferred into the inorganic form by the activity of the bone phosphatase during the last half. Thus the lipoid phosphorus is transformed into a suitable substrate for the bone phosphatase, reinforcing the small amount of the substrate initially present, and then, as calcification proceeds, is deposited in inorganic form. Kay made some observations on rabbit embryos and young animals, from which the graph in Fig. 377 (*a*) has been con-

structed. The kidney phosphatase increases in activity before birth, and afterwards attains a constant level—the bone phosphatase decreases to a constant level.

Fig. 377 (*b*) shows the admirable experiments of Fell & Robison who studied the phosphatase in chick embryo bones, developing both *in vivo* and *in vitro*. It is evident that the activity of the enzyme (as measured by the phosphorus hydrolysed in 24 hours from sodium glycerophosphate) increases in the bones from chicks incubated normally. The same increase is seen in bone fragments differentiating

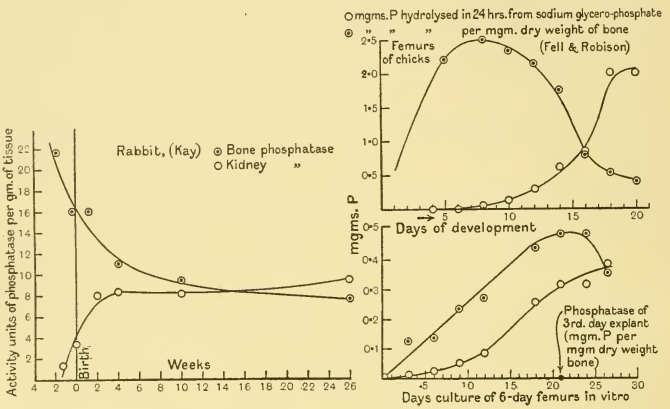



Fig. 377.

*in vitro* although it is slower. In both cases, a peaked curve is seen when the units of activity are referred to unit dry weight, no doubt because after a time the ash is laid down more rapidly than the phosphatase is formed. The almost perfect self-differentiation of the bones in these experiments was very striking; it was shown by 6th-day but not by 4th or 5th-day bones. In this connection the finding of phosphatase by Martland & Robison only in centres of active ossification in human foetal bones should be remembered.

It is worth remarking in connection with ossification that Hatchett in 1800 made the curious observation that "in the ova of those tribes of animals, the embryos of which have bones, there is a portion of oily matter, and in those ova whose embryos consist

entirely of soft parts, there is none. Hence it is concluded that a certain portion of oil is necessary for the formation of bone". What Hatchett actually did we shall never know, for nothing but the bare statement given above is contained in the place referred to by Prout, and that not in a paper by Hatchett himself but in one by Sir Everard Home. But in spite of our ignorance of Hatchett's technique, one cannot help surmising that he had attained in some measure, however feeble, an estimate of the lipoidal constituents in eggs of different species, and that, in actual fact, more of these substances are present in what Prout would call "the recent egg" where bones, with their calcium phosphate, have to be formed than where they have not. A study of Table 30 does not, unfortunately, lend weight to this view, but the analyses of eggs which included reliable estimations of lipoid phosphorus have been so few that no conclusion, either in favour of Hatchett or against him, can at present be drawn from them.

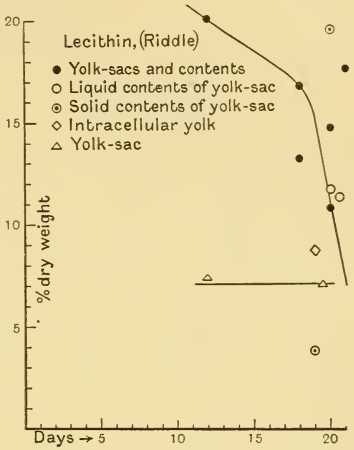


Fig. 378.

Other investigations besides those of Plimmer & Scott and Masai & Fukutomi have been made of the phosphorus in the hen's egg. Thus Riddle estimated the ether-soluble phosphorus in the yolk and yolk-sac of the chick during the last half of incubation, and, expressing his results in terms of lecithin as per cent. of the dry weight, obtained figures from which Fig. 378 has been constructed. An extremely marked absorption of phosphatides from the yolk is indicated during the last week of incubation, for the percentage phosphatides to dry weight in the yolk falls with a rush, and this is just what one would expect from the evidence in Plimmer & Scott's experiments. The phosphatide-content of the yolk-sac seems to remain stationary, if anything can be deduced from two points only, and the composition of the more solid parts of the yolk would seem

to be very variable. Riddle is certainly right in saying that after the 12th day the phosphatides are utilised more rapidly than the neutral fats, and the neutral fats more rapidly than the proteins. Riddle also found that yolk being absorbed from the follicle which secreted it in the ovary shows a more rapid utilisation of the phosphatides than of the neutral fats, but this may hardly be more than a coincidence. Iljin in his turn estimated the lecithin and residual protein phosphorus in the yolk only, before and after development, obtaining the following figures:

| | Phosphorus in milligrams per egg | |
|---|---|---|
| | Protein phosphorus | Lecithin phosphorus |
| Yolk at beginning ... ... | 150 | 65 |
| "Spare yolk" at end ... ... | 32 | 17 |
| Absorbed by embryo... ... | 118 | 38 |

Iljin's protein phosphorus figures do not correspond very well with those of Masai & Fukutomi, or of Plimmer & Scott, but his ether-soluble phosphorus ones do. Something must certainly have gone wrong with Iljin's vitellin analyses.

It was left for Cahn to make a proper series of lipoid phosphorus determinations on the embryo during its development. The results of his work were very interesting. Whether his absolute quantities were in good agreement with the figures of Plimmer & Scott cannot be stated, as the latter workers unfortunately omitted to give any absolute figures, confining their statements to percentages of the total phosphorus. Cahn found, as might be expected, that the total amount of lecithin phosphorus in the embryo rose with the growth of the body, but regularly, that is to say, not remaining very low till the 15th day and then suddenly rising rapidly, like the neutral fat. This difference in shape of the curves can be seen from Fig. 379, which is taken from his paper. When the lipoid phosphorus was related to wet and dry weight, however, more interesting curves appeared, as shown in Fig. 380. In per cent. of the moist tissue one finds a regular augmentation until the 15th day, after which a plateau supervenes up to hatching, though afterwards the curve again rises, and a chick 2 days after hatching contains as much as 660 mgm. per cent. wet weight. Then, as might be anticipated from a plateau on a wet weight curve, the dry weight curve rises to a peak, and thereafter falls away. This peak occurs, in the case of the

lipoid phosphorus, on the 10th day of development, and it is probable that it simply represents the impingement of the law of cephalocaudal

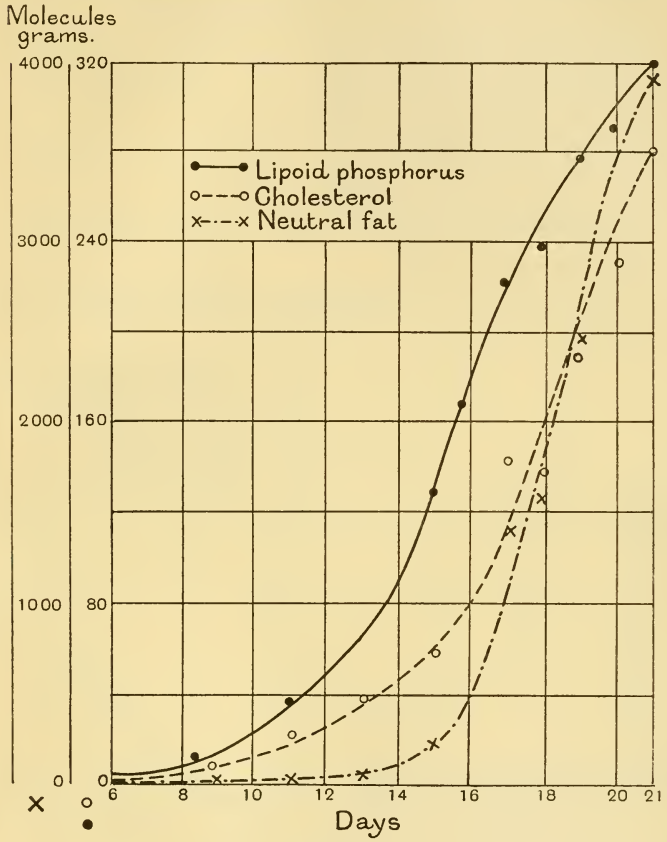



Fig. 379.

differential growth on the partition of solid substances in the body. If the central nervous system continued to occupy all through development the important ponderal position which it occupies in the first

10 days, the lipoid phosphorus would continue to rise in per cent. of the dry weight, but this is not the case, and the 10th day peak represents the point at which the cephalic end of the embryo ceases to dominate the chemical composition of the embryo. Cahn himself was more interested in the relation between water and lipoid phosphorus. In Fig. 381, the water-content of the embryo per 100 gm. is placed on the same graph as the water-content expressed in terms of grams per 100 mgm. of phosphatide phosphorus. It is evident that the two curves descend without evincing any close

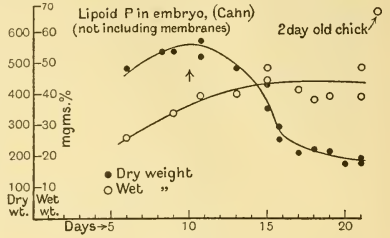



Fig. 380.

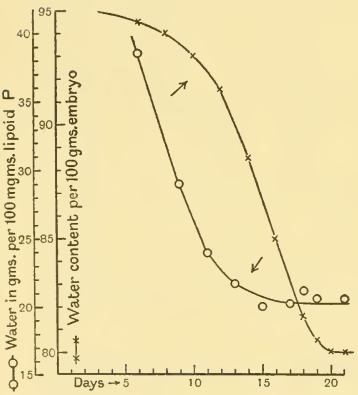



Fig. 381.

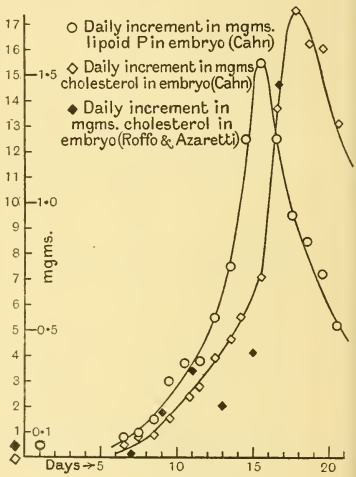



Fig. 382.

relationship, for the concavity of one lies towards the abscissa and that of the other towards the ordinate. Cahn next calculated the curve for daily increment of lipoid phosphorus, and found it to show (Fig. 382) an augmentation up to the 15th day, followed by a fall. This cor-

responds in an interesting way with the data of Plimmer & Scott, for it looks as if the daily accretion of lipoid phosphorus in the embryo rises until a point at which great quantities of the lipoid of the yolk begin to be decomposed, and then falls off. Cahn thought that all the constituents of the embryo would show peaks on the daily increment curves, but, as may be seen from many of the graphs in this book, in actual fact this is not the case. He interpreted the peaks which do occur as being evidence of S-shaped curves of absolute magnitude. But, as we have seen, not all such curves are S-shaped in the time of the chick's development. Cahn finally calculated the percentage growth-rate of the lipoid phosphorus, and found that it followed the usual curves for percentage growth-rates of individual substances (see Fig. 364).

## 12·2. Tissue Phosphorus Coefficients

The conception of tissue coefficients, or ratio values constant for a given tissue for a given animal of a given age, first introduced by Mayer & Schaeffer, is interesting in this connection.

The ratio:

$$\frac{\text{Total fatty acids}}{\text{Lipoid phosphorus}},$$

can be regarded roughly as a measure of the relative amounts of lipoid fatty acids and triglyceride fatty acids in the tissue. Mayer & Schaeffer obtained the following figures in the course of their experiments, all on the adult animal:

| | Total fatty acids/lipoid phosphorus | | | | |
|---|---|---|---|---|---|
| | Liver | Kidney | Lung | Pancreas | Muscle |
| Man ... ... | 36 | 25 | — | 55 | 213 |
| Dog ... ... ... | 21 | 22 | 22 | 27 | 69 |
| Rabbit ... ... | 20 | 22 | 25 | — | 19 |
| Guinea-pig ... ... | 16 | 25 | 19 | — | 38 |
| Pigeon ... ... | 33 | 27 | 16 | — | 53 |
| Eel ... ... ... | 126 | 34 | — | — | 288 |

Thus only two tissues, the liver of the guinea-pig and the lung of the pigeon, had so little an amount of neutral fat that the ratio fell below 17, though in four or five cases it fell to or below 20. On the other hand, in certain cases the muscle showed an immense preponderance of neutral over lipoidal fatty acids. What happens in the chick embryo during its development? Cahn calculated this ratio from his data, with the result that it turned out to be well below the very lowest of the adult values. In other words, the fatty acids of the

embryo, minute in amount as they are during the first 10 days, must be almost wholly combined in the lipoid molecule, and only towards the end of development, when the fat content is beginning to rise sharply, does the coefficient rise too, showing an accumulation of neutral fat. Presumably by shortly after hatching the ratio would approach one of the lower levels seen in the table given above. Cahn's figures for the ratio are plotted in Fig. 383.

Javillier & Allaire later suggested the use of the following coefficients:

$$\frac{\text{Purine phosphorus}}{\text{Lipoid phosphorus}} \times 100,$$

and

$$\frac{\text{Total phosphorus} - (\text{lipoid phosphorus} + \text{purine phosphorus})}{\text{Total phosphorus}} \times 100,$$

which latter is equivalent to

$$\frac{\text{Inorganic phosphorus} + \text{water-soluble organic phosphorus}}{\text{Total phosphorus}} \times 100.$$

It is instructive, however, to compare the data of Javillier & Allaire, and of Javillier, Crémieu & Hinglais, for various adult tissues, with the data at our disposal for the foetal tissues of the chick and the undeveloped egg, more especially as Javillier & Crémieu have investigated in this way the tissues of various invertebrates, and Javillier, Allaire & Rousseau the tissues of the whole white mouse from birth to 40 days of post-natal life. Javillier & Crémieu, in comparing the invertebrate tissues with those of mammals, suggested using the total phosphorus minus the inorganic phosphorus as the denominator, instead of the true total phosphorus, a procedure which they hoped would make a better basis of comparison. This non-calcification phosphorus they

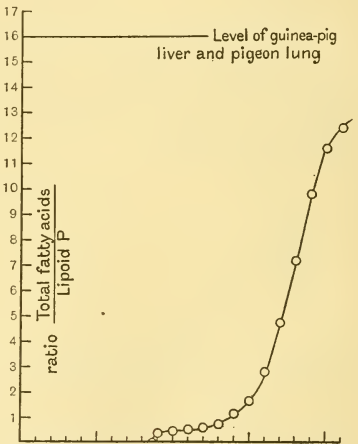



Fig. 383.

Table 175. *Distribution of phosphorus in tissues.*

| Material | Nuclein phosphorus / Total phosphorus × 100 | Nuclein phosphorus / Lipoid phosphorus × 100 | Lipoid phosphorus / Total phosphorus × 100 | Inorganic phosphorus / Total phosphorus × 100 | Stable organic water-soluble phosphorus / Total phosphorus × 100 | Nuclein phosphorus / Active phosphorus × 100 | Lipoid phosphorus / Active phosphorus × 100 | Stable organic water-soluble phosphorus / Active phosphorus × 100 | Investigator | |
|---|---|---|---|---|---|---|---|---|---|---|
| Coelenterate (*Sagartia parasitica*) ... | 10·7 | 38·5 | 27·8 | 35·1 | 26·2 | 16·5 | 42·9 | 40·5 | Javillier & Crémieu | |
| Holothurian (*Synapta inhaerens*) ... | 10·7 | 29·9 | 35·7 | 29·2 | 24·5 | 15·1 | 50·4 | 34·5 | ,, | ,, |
| Polychaete worm (*Nereis diversicolor*) ... | 6·9 | 20·7 | 33·6 | 16·9 | 42·6 | 8·4 | 40·4 | 51·2 | ,, | ,, |
| Oligochaete worm (*Tubifex rivulorum*) | 11·4 | 36·2 | 31·5 | 14·1 | 43·0 | 13·2 | 36·6 | 49·9 | ,, | ,, |
| Leech (*Hirudo medicinalis*) ... ... | 6·9 | 15·9 | 43·2 | 20·7 | 29·2 | 8·7 | 54·5 | 36·3 | ,, | ,, |
| Lamellibranch mollusc (*Cardium edule*) | 12·2 | 42·5 | 28·7 | 20·6 | 38·5 | 15·4 | 36·1 | 48·5 | ,, | ,, |
| Insect (*Hydrophilus piceus*) ... ... | 12·1 | 40·7 | 29·8 | 7·4 | 50·7 | 13·0 | 32·2 | 54·7 | ,, | ,, |
| Teleostean fish (*Cyprinus carpio*) ... | 2·4 | 57·0 | 4·2 | 91·3 | 2·1 | 27·5 | 48·3 | 24·1 | ,, | ,, |
| Mammal (*Mus domesticus*) ... ... | 3·6 | 37·7 | 9·5 | 79·2 | 7·7 | 17·2 | 45·7 | 37·0 | ,, | ,, |
| Mammal (*Mus rattus*) ... ... ... | — | — | 8·0 | — | — | — | — | — | Cahn | |
| Rabbit, kidney ... ... ... ... | 25 | — | — | — | — | — | — | — | ,, | |
| muscle ... ... ... ... | 13 | — | 17 | — | — | — | — | — | ,, | |
| intestine ... ... ... | 37 | — | 26 | — | — | — | — | — | ,, | |
| liver ... ... ... ... | 26 | — | 47 | — | — | — | — | — | ,, | |
| Guinea-pig, liver ... ... ... | 29 | — | — | — | — | — | — | — | ,, | |
| Dog, muscle ... ... ... ... | 9 | — | 25 | — | — | — | — | — | ,, | |
| intestine ... ... ... ... | 31 | — | 29 | — | — | — | — | — | ,, | |
| liver ... ... ... ... | 21 | — | 40 | — | — | — | — | — | ,, | |
| kidney ... ... ... ... | 23 | — | 50 | — | — | — | — | — | ,, | |
| lung ... ... ... ... | 29 | — | 46 | — | — | — | — | — | ,, | |
| Horse, thymus ... ... ... ... | 58 | 310 | 19 | | 23 | — | — | — | Javillier & Allaire | |
| pancreas ... ... ... | 37 | 90 | 40 | | 23 | — | — | — | ,, | ,, |
| spleen ... ... ... ... | 35 | 109 | 33 | | 33 | — | — | — | ,, | ,, |
| liver ... ... ... ... | 19 | 38 | 51 | | 30 | — | — | — | ,, | ,, |
| lung ... ... ... ... | 19 | 37 | 52 | | 28 | — | — | — | ,, | ,, |
| thyroid ... ... ... ... | 32 | 93 | 34 | | 34 | — | — | — | ,, | ,, |
| kidney ... ... ... ... | 15 | 33 | 46 | | 38 | — | — | — | ,, | ,, |
| heart ... ... ... ... | 6 | 12 | 50 | | 44 | — | — | — | ,, | ,, |
| brain ... ... ... ... | 5 | 6 | 79 | | 16 | — | — | — | ,, | ,, |
| muscle ... ... ... ... | 3 | 9 | 32 | | 66 | — | — | — | ,, | ,, |
| spinal cord ... ... ... | 1 | 1 | 90 | | 8 | — | — | — | ,, | ,, |
| Hen's egg, undeveloped ... ... | 2 | 3 | 65 | | 33 (phospho- | — | — | — | Plimmer & Scott; Cahn | |
| Chick embryo, 14th day ... ... | 21 | 100 | 22 | | 56 protein) | — | — | — | and others | |
| 17th day ... ... | 27 | 150 | 18 | | 55 | — | — | — | ,, | ,, |
| 19th day ... ... | 18 | 120 | 15 | | 67 | — | — | — | ,, | ,, |
| 21st day ... ... | 12 | 63 | 19 | | 69 | — | — | — | ,, | ,, |
| White mice. Days from birth 0 ... | 14 | 114 | 12 | 65 | 9 | 40 | 35 | 25 | Javillier, Allaire & Rousseau | |
| ,, 7 ... | 11 | 72 | 16 | 60 | 12 | 29 | 40 | 31 | ,, | ,, |
| ,, 14 ... | 7 | 50 | 14 | 70 | 9 | 23 | 46 | 31 | ,, | ,, |
| ,, 21 ... | 7 | 46 | 16 | 64 | 12 | 20 | 44 | 35 | ,, | ,, |
| ,, 30 ... | 11 | 56 | 19 | 59 | 11 | 26 | 46 | 27 | ,, | ,, |
| ,, 40 ... | 10·5 | 57 | 18 | 61 | 10 | 27 | 47 | 26 | ,, | ,, |
| Pigeon (adult), liver ... ... ... | 23·7 | 57·5 | 41·3 | — | 25·7 | — | — | — | Javillier, Crémieu & Hinglais | |
| kidney ... ... ... | 8·8 | 19·4 | 46·0 | — | 29·4 | — | — | — | ,, | ,, |
| brain ... ... ... | 5·8 | 8·3 | 69·7 | — | 14·8 | — | — | — | ,, | ,, |
| heart ... ... ... | 5·6 | 11·7 | 48·4 | — | 35·2 | — | — | — | ,, | ,, |
| muscle ... ... ... | 3·0 | 8·4 | 35·8 | — | 51·4 | — | — | — | ,, | ,, |

The active phosphorus is the total phosphorus minus the inorganic phosphorus; the effect of bones on the figures is thus avoided.

called the "active phosphorus". As Table 175 shows, the mammals and invertebrates can hardly be compared except in this way, but when it is done it is found that the mammal comes intermediate between some of the invertebrates as regards its phosphorus distribution. It does not seem possible to draw any ontogenetic conclusions from this body of data, but it is as yet, of course, very restricted.

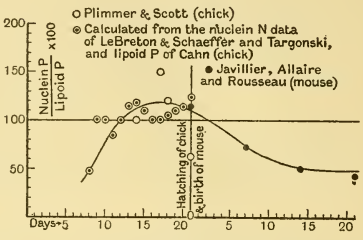



Fig. 384.

The undeveloped egg of the bird has, as would be expected, a phosphorus distribution very different from any tissue, and even the body of the 14-day chick is not very like any of the adult tissues of the bird (we only have figures for the pigeon). The nuclein phosphorus/lipoid phosphorus ratio is interesting. The values for the chick placed in Table 175 are taken from Plimmer & Scott's figures for the phosphorus distribution in the embryo, and these authors, as I have before remarked, did not give their figures in milligrams per embryo, or even per cent. of the weight of the material. Thinking therefore that a calculation based on percentages of the total phosphorus alone might be distorted, I calculated the nuclein phosphorus in the embryo (which has never been directly estimated) from the data of LeBreton & Schaeffer and Targonski for the nuclein nitrogen in the embryo, and in this way obtained the nuclein phosphorus/lipoid phosphorus ratio for each day during the last fortnight of development. The result, given in Fig. 384, shows first a rise and then the beginning

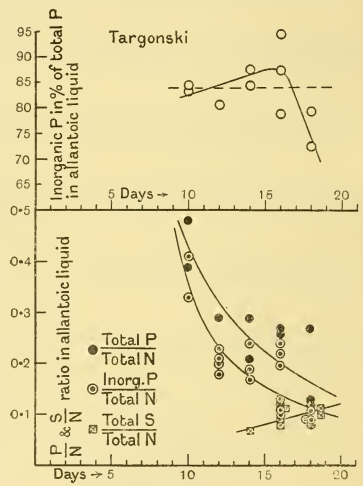



Fig. 385.

of a fall. This fits in with the data for the mouse, as appears from the fact that in Javillier, Allaire & Rousseau's figures for the phosphorus distribution in white mice, the nuclein phosphorus/lipoid phosphorus ratio is above 100 at birth, and then falls. Apparently the nuclein phosphorus never greatly exceeds the lipoid phosphorus.

So far nothing has been said about the phosphorus in the allantoic liquid of the chick. It has indeed only been estimated by one worker, namely, Targonski. By measuring the total and inorganic phosphorus in the allantoic liquid during the last half of development, he was able to find the percentage of the total allantoic phosphorus existing there in inorganic form, and the ratio:

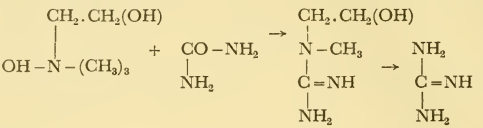
$$\frac{\text{Total phosphorus}}{\text{Total nitrogen}}.$$

The main results are plotted on Fig. 385. What happens to the inorganic phosphorus in per cent. of the total phosphorus is not very clear; it might be regarded as showing a distinct break at the 16th day, but more probably the relation remains constant. The phosphorus/nitrogen ratios, however, are very interesting, for both the total phosphorus/total nitrogen and the inorganic phosphorus/total nitrogen decline steadily, the former rather less rapidly than the latter. The phosphorus/nitrogen ratio never reaches that for vitellin, namely, 0·057. In other words, there is always more phosphorus in the allantoic liquid, and sometimes much more, than would result from a simple removal of part of a vitellin molecule (if it was homogeneous) and excretion of the results of its combustion. These problems need much further investigation.

## 12·3. Choline in Avian Development

If now we return to the consideration of Plimmer & Scott's curves for phosphorus distribution throughout the egg, it will be remembered that the ether-soluble phosphorus diminishes greatly, i.e. that the lipoids of the yolk, lecithin and kephalin, are broken down, releasing inorganic phosphorus. The other substances released at the same time are worth consideration. From its formula, one would expect that a good deal of free choline would make its appearance during incubation. The amount of choline in the hen's egg has been studied by Sharpe and by Okada. The former investigator thought that the increase in total guanidine during incubation might be due to a decrease

in total choline, and made a few estimations by means of his own method to see whether this was so. Choline, by reacting with urea, might be the precursor of guanidine, and the formulae of the three substances lent a certain colour to the suggestion:



Sharpe's results are seen in Fig. 386, from which it is clear that the total choline content of the hen's egg diminishes from 400 mgm. per cent. on the 1st day of development to 220 mgm. per cent. at the 21st. This decrease, argued Sharpe, meant a loss of total choline of 130 mgm.; and Burns found a rise in the amount of total guanidine per egg of from 80 to 260 mgm., i.e. an increase of 180 mgm. Although, on the basis of the transformation pictured above, 1gm. of choline should yield 2 gm. of guanidine, yet the correspondence was sufficient to justify Sharpe's remark that in all probability choline was here the precursor of guanidine.

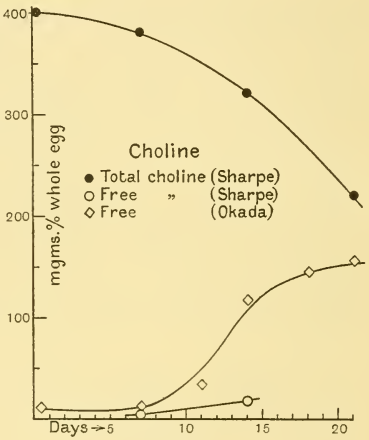



Fig. 386.

The shape of Sharpe's descending total choline curve cannot be emphasised, as it is constructed from so few points, but it may be remarked that it begins to fall much earlier than the lipoid phosphorus, either in the experiments of Plimmer & Scott or in those of Masai & Fukutomi. Is there here a possibility that the choline portion of the lecithin molecule may be detached before the phosphoric acid, so that the latter would remain ether-soluble?

The subject of choline in the egg was carried a stage further by Okada, who estimated the free choline only during development.

Sharpe had already published two estimations of this in his preliminary note, but Okada's curve amplified them, and demonstrated that, of the choline which is found in the egg at the end of incubation, more than two-thirds is uncombined in lecithin. The almost complete disappearance of the combined choline, seen in the work of Sharpe and of Okada, is a striking sidelight on the utilisation of the lipoids of the egg by the chick. It has since been confirmed by Fukutomi, but his figures are unfortunately not published. From the data of Sharpe and Okada, the combined choline can be calculated; this corresponds to 386 mgm. per cent. at 0 day, and 65 mgm. per cent. just before hatching. As there are about 187·8 mgm. per cent. of total phosphorus at the beginning and 62 per cent. of this is lecithin phosphorus (Plimmer & Scott), there must be 115·2 per cent. of lecithin phosphorus, i.e. 2880 mgm. of lecithin, and therefore 390 mgm. per cent. of choline—almost exactly what was obtained in Sharpe's direct estimation.

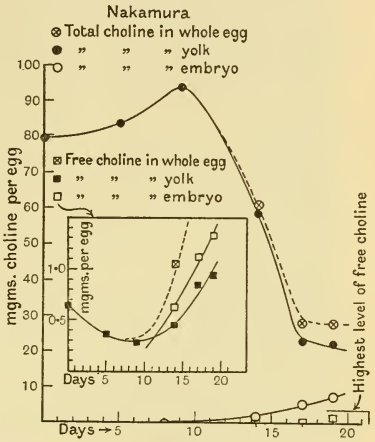



Fig. 387.

Unfortunately, the more recent investigations of Nakamura have not supported this apparently straightforward story, for, using a simple gravimetric method, in which the melting-point and other properties of the crystals were verifiable, he obtained results in some opposition to those already described. Fig. 387 shows them. The total choline in the whole egg, according to him, rises by 10 or 15 per cent. from the initial value to a maximum of just over 90 mgm. per egg at the 9th day, after which it rapidly falls off, reaching what seems to be a steady level about the 18th day (of about 25 mgm. per egg). This contrasts with the Sharpe-Okada-Fukutomi results, according to which the whole egg contains some 200 mgm. of total choline before incubation, and some 110 mgm. at the end of it. While in both cases there is a large fall according to Nakamura the

free choline never reaches more than 3 mgm. in the whole egg by the end of development, whereas according to Okada it reaches at least 75 mgm., and according to Sharpe at least 15. It is difficult to see to what these large discrepancies can be due, but it is probable that the colorimetric method used by the other observers may be less satisfactory than the gravimetric one used by Nakamura. If, however, we are to accept Nakamura's figures as the best, we have to take seriously the initial rise which he found in the total choline, and neither he nor anyone else has been able to suggest anything to explain it. The egg-white and the amniotic and allantoic liquids were worked up for choline by Nakamura, but he never succeeded in finding any there. Fig. 388, which gives his results in per cent. wet weight of the yolk and the embryo, seems to show that, whereas the free choline of the yolk has large fluctuations, that of the embryo remains at a constant figure, and that the total choline declines in the yolk and rises in the embryo. Nakamura made no speculations as to the fate of the 65 mgm. of combined choline lost from the egg during its development, except to suggest that it might provide the creatinine of the embryo. We have seen, however, in Section 10 that the highest estimate of the creatine or total creatinine content of the embryo by the time of hatching is 25 mgm., so here also there is some discrepancy.

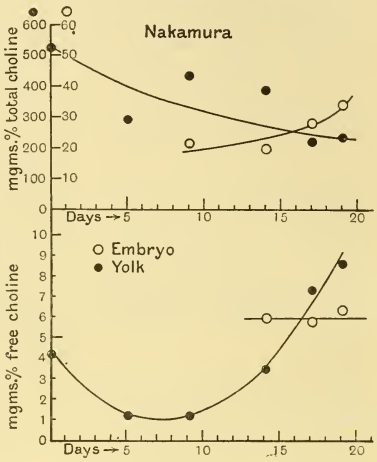



Fig. 388.

## 12·4. The Metabolism of Sterols during Avian Development

We can now pass to the cholesterol metabolism of the egg. This substance was prepared from the yolk by Lecanu in 1829, and studied by Gobley in 1846 (see Plate XII). In 1912 Hanes, while working with a chick embryo of 19 days' incubation, was attracted by the

YOLK OF HEN'S EGG AT THE SECOND DAY OF INCUBATION

Microphotographed by Dr V. Marza through crossed Nicol prisms (31°). The doubly-refracting esters of cholesterol are seen to be localised at the periphery of the vitelline globules. Magnification 8 × A.

brilliant yellow colour of the liver, and, teasing some of it out, examined it between the crossed Nicol prisms of a polarising microscope. The result was a very large number of droplets showing double refractivity. On adding Sudan III, Hanes observed that the droplets stained a deep yellowish red, from which he concluded that the chick's liver contains a great deal of cholesterol esters towards the end of incubation. This led him to make a histochemical study of the liver throughout the incubation period. As the liver grows, he said, it assumes more and more the colour of the yolk. The liver cells of the 6th-day chick contain numerous small fatty globules, but under the polarising microscope these are isotropic. The globules gradually increase in size and number, and about the 14th day of incubation many of them begin to show double refraction. By the time of hatching the liver is very rich indeed in fat droplets, recalling Imrie & Graham's work on guinea-pig livers. Hanes then availed himself of Kawamura's critical study of the differential histochemical methods for identifying the various fatty substances, and applied them to the chick livers. His results were as follows:

| | Liver from 6th to 14th day | Liver from 14th to 21st day |
|---|---|---|
| Nicol prisms ... ... ... | Isotropic | Anisotropic |
| Sudan III ... ... ... | Yellowish red | Yellowish red |
| Nile blue sulphate ... ... | Faintly blue | Uncoloured or faintly pink |
| Smith's stain ... ... ... | Dark bluish black | Faintly bluish grey or uncoloured |
| Ciaccio's stain ... ... | Positive | Negative |
| Fischler's stain ... ... | Negative | Negative |
| Neutral red ... ... ... | Negative | Negative |
| Salkovski's test for cholesterol | Faintly positive | Strongly positive |

He concluded from these facts that the fat present in the chick liver up to the 14th day of incubation is not pure neutral fat, nor at that time are any soaps or free fatty acids present. This agrees exactly with Mayer & Schaeffer's ratio mentioned above (p. 1212). Moreover, Smith's stain, which does not colour neutral fats or cholesterol esters, and does colour lecithin-like substances, is very positive in the early stages. The same remarks apply to Ciaccio's method. As incubation proceeds, the droplets in the liver gradually cease to react with Smith's stain, and by 4 or 5 days after hatching this method will not colour them at all. Ciaccio's stain is likewise negative during the last week of incubation, and after hatching. But it is precisely during this time that they give the reactions characteristic of cholesterol esters. Thus, if warmed to about 40°, they lose their

doubly refracting property, and upon cooling they again become anisotropic. Cholesterol esters are remarkably resistant to autolytic change, and this was found by Hanes to be a property of the droplets in the liver also. They retained their anisotropism for 10 days when autolysed at 37°, and at last changed to long needle-like crystals, which showed the reversible anisotropism on heating. Hanes concluded that during development esters of cholesterol appeared in the chick's liver and the lipoids disappeared. He correlated these changes with ossification and the arrival of calcium for deposition in the bones as calcium phosphate, drawing attention to the fact that if, as Plimmer & Scott had demonstrated, the lecithin of the yolk was broken down to provide the inorganic phosphorus, something must happen to the fatty acids of the lipoid molecule. The lecithin, said Hanes, must be absorbed gradually by the vitelline vessels, and carried to the liver, together with neutral fat, cholesterol, vitellin, etc. The lecithins then breaking down to yield glycerophosphoric acid, the latter or some closely related substrate for Robison's bone enzyme travels to the bones, and the phosphorus being liberated, is deposited as calcium phosphate. Meanwhile, the two fatty acid molecules left by the decomposition of the lecithin would esterify some or all of the cholesterol which had also been absorbed by the vitelline vessels. The only weak point about this scheme was that, if cholesterol was going to be esterified in the liver, why should it ever have been free, in view of the great amounts of fatty acids everywhere in the egg? However, no doubt Hanes had in mind some special activation of the cholesterol molecule which could take place only in the liver. He found that the cholesterol ester droplets disappeared from the liver after hatching, though a fortnight later they were still abundant, and they were not present in the liver of the adult hen.

Hanes also examined the fat droplets of the yolk-sac. Here he found a good many with the property of double refraction, but they had other characteristics which sharply distinguished them from the anisotropic droplets of the liver. Thus they did not lose the property when heated to 90°, they stained with neutral red, and exhibited myelin forms upon the addition of water. Nile blue sulphate stained them blue. They behaved in every way, in fact, like kephalin or sphingomyelin. However, at the time of hatching, the yolk-sac, now within the body of the chick, showed fatty droplets

which gave all the reactions of cholesterol esters. Hanes suggested that finally the yolk-sac, like the liver, takes part in the decomposition of the lecithin molecule. Finally, he recalled that Windaus had reported the presence of a great deal of esterified cholesterol in areas of pathological calcification, such as atherosclerotic aortas. He mentioned that in examining the livers of foetal dogs and of new-born puppies he had found a large quantity of anisotropic droplets. On the other hand, he failed to find any in the liver of the foetal pig (stage of development not stated).

Apparently Hanes was unaware of some work of Valentin published as early as 1871. Valentin investigated the doubly refracting droplets in chick embryos, and stated that none of the tissues of the 3rd, 4th, 5th or 6th day embryos showed them, but that on the 7th day a very few appeared. He obtained comparable results in a study of the embryonic liver of the frog. Chalatov also had reported anisotropic globules in liver cells of rabbits fed with egg-yolk for 5 months. After Hanes' report, work was continued on much the same lines by Yamaguchi, who examined the livers of the foetuses of man, the rabbit, dog, guinea-pig, bat, toad, snake and salmon. In the two latter cases no cholesterol esters ever appeared as in all the others. In the human foetal liver, the esters appeared about the end of the 1st month, and increased in amount—as far as histochemical work could show—until the beginning of the 5th month, after which they disappeared.

Chemical estimations of free and combined cholesterol in the hen's egg during development have, generally speaking, confirmed the views of Hanes, and indeed widely extended them. Its absorption from the yolk was studied by Idzumi, who simply estimated the amount of unsaponifiable substance in the petrol-ether extract. It is impossible to decide what his data mean, for he obtained much more total unsaponifiable substance when he estimated the embryo and the remainder separately than when he estimated the whole egg. The work of Mueller was on a different level, but, before considering it, it will be convenient to discuss the question of whether cholesterol is synthesised at all by the developing chick embryo. To settle this it was only necessary to estimate the total cholesterol present at the beginning of development, and to compare it with the total cholesterol present at the end. The first workers to do this were Ellis & Gardner in 1908. They pounded up the eggs and embryos in mortars with plaster of paris, and allowed the mass to set, after which they powdered it, and

extracted it for 12 days with ether in a Soxhlet. The cholesterol in the unsaponifiable fraction was estimated gravimetrically as cholesterol benzoate. They found that no increase took place during development, the cholesterol accounting for 382·7 mgm. per cent. of the wet weight of the egg, and for 369·3 mgm. per cent. of the wet weight of the hatched chicks, or 317·2 mgm. per cent. expressed in terms of the original weight of the eggs. At first sight it would seem as if there had occurred a loss of cholesterol from the egg, but Ellis & Gardner pointed out that the difference between the average percentage in eggs and in chicks, i.e. 66 mgm., was of much the same order of magnitude as the average deviation from the mean in the two cases, i.e. 57 mgm. for the eggs and 75 mgm. for the chicks. So, as they only analysed 8 eggs and 8 chicks, and as they were not very confident in their method, they preferred to conclude that in all probability neither loss nor gain took place during the incubation of the egg. In another experiment in which 6 eggs and 6 chicks were analysed together, 489 mgm. per cent. were obtained in the former case, and 467 mgm. per cent. in the latter.

This result substantiated the view held in all their investigations by Ellis & Gardner, namely, that cholesterol cannot be synthesised by the animal body. However, Channon and Dam demonstrated some years later that the reverse proposition is true for the chick after hatching, and it was not long before Thannhauser & Schaber, using Windaus' method, reported an increase of total cholesterol during the incubation period. Their data were as follows:

| | Free cholesterol | | Cholesterol esters | | Total cholesterol | |
|---|---|---|---|---|---|---|
| Days | Milligrams | % dry weight | Milligrams | % dry weight | Milligrams | % dry weight |
| 0 | 173 | 1·316 | 54·2 | 0·417 | 227·2 | 1·717 |
| 21 | 128 | 1·065 | 123·5 | 1·027 | 251·5 | 2·09 |
| Change | −45 | −0·251 | +69·3 | +0·61 | +24·3 | +0·35 |

Thus the free cholesterol decreased by 26 per cent. of the initial value, the combined cholesterol increased by 128 per cent. of the initial value, and the total cholesterol, thus partially compensated, increased by 10·7 per cent. of its initial value. We see here clearly that production of cholesterol esters observed histochemically by Hanes. Roffo & Azaretti, the next workers on the sterols of the egg, found no marked change in the cholesterol content of the whole egg before and after incubation, there being 219 mgm. per egg at

the beginning of development (1·86 per cent. of the dry weight) and 282 mgm. per egg at 18 days. The admitted positive balance was probably not significant. The later work of Dam was designed to answer this question. In one of her experiments, the cholesterol per egg was 245·0 mgm., and that of the 21st-day embryos 263·5 mgm.; in another series, that of the undeveloped eggs was 310·0 mgm. and of the hatching chick 343·0 mgm. In the former case the increase

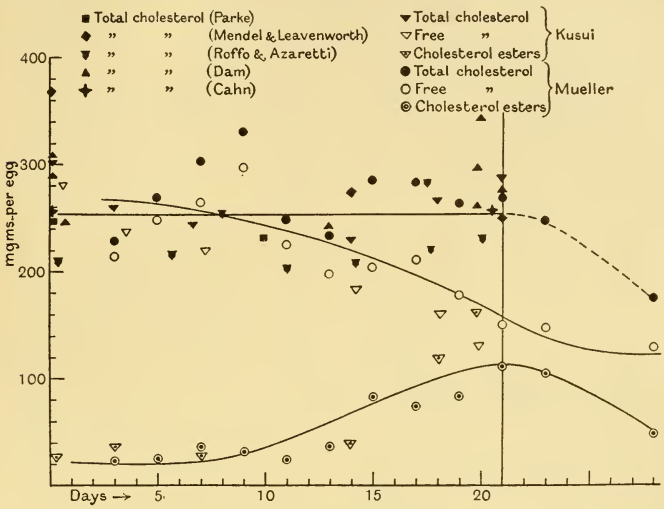



Fig. 389.

on the initial value was 7·6 per cent. and in the latter case 10·6 per cent., and these amounts, though not considerable, were of the same order as those found by Thannhauser & Schaber, and were invariably found by Dam. In a subsequent paper, however, she gave further figures which showed no increase.

Analogous results to these were obtained by Mueller in 1915, who investigated the free and combined cholesterol content of the whole egg throughout incubation. From his figures, which are plotted in Fig. 389, it can be seen that the total cholesterol in the egg remains at a steady level throughout incubation. But the most

striking thing about the graph is the way in which the free cholesterol diminishes up to hatching, and the cholesterol esters increase. Thus on the 3rd day of development, the free cholesterol makes up 89·9 per cent. of the total cholesterol, but on the 21st only 58·68 per cent. Nothing could fit in more strikingly with the results of Hanes. As for the question of a synthesis of cholesterol, Mueller's data do not show any such process to have taken place, but an increase of only 11 per cent. might, of course, be masked in any but the most careful researches specially designed to test the point. The significance of the fall in total cholesterol found by Mueller after hatching is not evident, but the fact that the cholesterol esters diminish then is in admirable agreement with Hanes' work. Mueller suggested that the bile acids might be formed from cholesterol, and this would explain Ellis & Gardner's decrease, but the process could hardly be operative on the basis of his own results and those of Thannhauser & Schaber. Mueller, in order to carry further the suggestions of Hanes, investigated some embryonic livers separately, and reported that by no means all the cholesterol esters were contained in the liver, nor was the cholesterol of the liver all in the combined form. Five 20th-day livers were analysed together, yielding 17·9 mgm. of free cholesterol and 51·6 mgm. of combined cholesterol, while in the combined bodies of the 5 embryos, plus the yolk-sac, still partially unabsorbed, there was about 300 mgm. of combined cholesterol. Mueller largely agreed with Hanes' theory, and went so far as to speak of a detoxication of the lipoid fatty acids by combination with the cholesterol (why should the released fat be toxic?), but he calculated that a gram of lecithin would produce much more free fatty acid than could be esterified by 100 mgm. of cholesterol. It is not likely that the explanation of the whole process lies in a "detoxication", although there is evidence, such as the

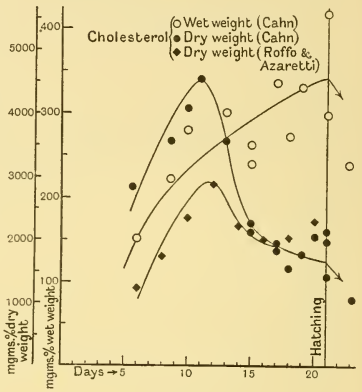



Fig. 390.

experiments of Robertson, that a definite cholesterol-lecithin balance is necessary for the normal functioning of the systems in the living organism. This notion is akin to that contained in Mayer & Schaeffer's cholesterol/lipoid phosphorus ratio, which will presently be discussed. Mueller's findings were later fully confirmed by Dam who found 12 per cent. of the cholesterol to be esterified at the beginning of development, but 45 per cent. at the time of hatching, and by Kusui, who could observe no effect of injected adrenalin on the percentage of esters.

Estimations of the cholesterol in the whole egg were also made by Parke and by Mendel & Leavenworth—their results are included in Fig. 389. It was not until 1926 that estimations were made of the cholesterol in the embryonic body, namely by Roffo & Azaretti, using the method of Windaus. Two years later another set of data was obtained by Cahn (see Fig. 379). The two investigations agree well enough, and the rise is continual, following the growth of the embryo. When related to wet weight, however, Cahn observed a rise, falling off in speed with age, and when related to dry weight, a rise to the mid-point of development, followed by a fall (Fig. 390). Roffo & Azaretti's data show this too. We thus find that 100 gm. of dry weight of embryo contain more cholesterol on the 11th day of development than at any other time. Cahn next calculated the daily increments, and found that they fell on a curve having a peak about the 18th day of development. This curve is plotted in Fig. 382 beside that for daily increments of lipoid phosphorus. Cahn's curve for percentage growth-rate of cholesterol is shown in Fig. 364.

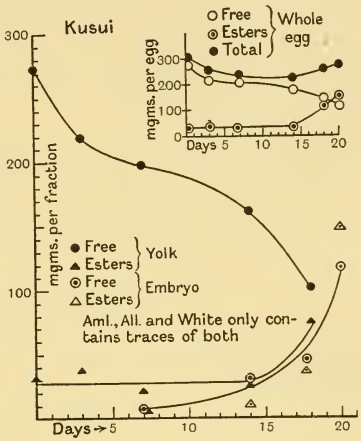



Fig. 391.

More recently Kusui has reinvestigated the cholesterol metabolism of the hen's egg, and has made a step forward by showing that except

for stray traces, it is confined to the yolk and the embryo, i.e. none appears in the white, the amniotic, or the allantoic liquid. His figures for total cholesterol in the whole egg, graphically reproduced in Fig. 391, show a diminution followed by a rise, but the dimensions of the change are not sufficient to warrant a belief in a decomposition followed by a synthesis. They come within the zone of other workers' results, shown in Fig. 389. Apart from this, Kusui confirmed the earlier discovery of a decrease of free and an increase of combined cholesterol. It would seem that the increase of cholesterol esters is not confined to the embryonic body but also takes place in the yolk.

According to Dam about 1·6 mgm. of cholesterol in each yolk is attached in some way to the proteins and cannot be removed by ether extraction unless they are first hydrolysed. No oxycholesterol exists in eggs or embryos.

## 12·5. The Relation between Lipoids and Sterols: the Lipocytic Coefficients

We are now in a position to consider the "lipocytic coefficients" of Mayer & Schaeffer. The first is:

$$\frac{\text{Total cholesterol}}{\text{Total fatty acids}} \times 100.$$

This was established by Mayer & Schaeffer for the following animals and tissues:

| | Electric organ | Lung | Kidney | Liver | Muscle |
|---|---|---|---|---|---|
| Dog ... ... ... | — | 20 | 10 | 7 | — |
| Rabbit ... ... | — | 17 | 13 | 8 | 2 |
| Guinea-pig ... ... | — | 15 | 8 | 6 | 7 |
| Pigeon ... ... | — | 24 | 9 | 8 | 2 |
| Eel ... ... ... | — | 11 | 7 | 4 | 2 |
| Torpedo ... ... | 22 | 12 | — | 1 | 1 |

Thus as a general rule the lung has much more cholesterol in relation to its total fatty acids than the muscle. Mayer & Schaeffer then tabulated the water-content of a number of tissues of different animals, and made the interesting discovery that a parallelism existed between water-content and lipocytic coefficient. Wherever the latter was high, so was the former, and *vice versâ*. This is illustrated by the following table:

| | gm. water associated with 100 gm. dry weight | | | | | |
|---|---|---|---|---|---|---|
| | Electric organ | Lung | Kidney | Liver | Muscle | Brain |
| Dog ... ... | — | 352 | 315 | 236 | 281 | — |
| Rabbit ... | — | 408 | 340 | 278 | 335 | 399 |
| Guinea-pig ... | — | 387 | 416 | 278 | 347 | — |
| Rat ... ... | — | — | — | — | — | 399 |
| Pigeon ... | — | 310 | 306 | 241 | 241 | — |
| Torpedo ... | 1112 | — | — | 70 | 445 | — |

The correspondences are by no means exact, and doubtless often obscured by the existence of other factors, but a general relationship seems clear. In a later paper, Mayer & Schaeffer studied the imbibition properties of tissues, and were finally able to reduce their data to the following expression:

$$\text{Maximum water retained by 1 gm. dry weight of tissue} \times \frac{\text{Total fatty acid}}{\text{Total cholesterol}} = K.$$

The constant was always about 60, and Mayer & Schaeffer compared this generalisation to Boyle's Law, but for the details reference must be made to their original papers. It is now possible to calculate the lipocytic coefficient for the chick through its development, and it is shown in Fig. 392. The earlier values are far higher than anything found in the adult, but by the end of development adult values are reached, as is shown by the horizontal lines drawn to represent the lipocytic coefficient in the organs of the full-grown pigeon. Now according to Mayer & Schaeffer's generalisation, the water-content of the embryo ought to be very high in the earlier stages, and should descend to nearly an adult level at hatching; and this, of course,

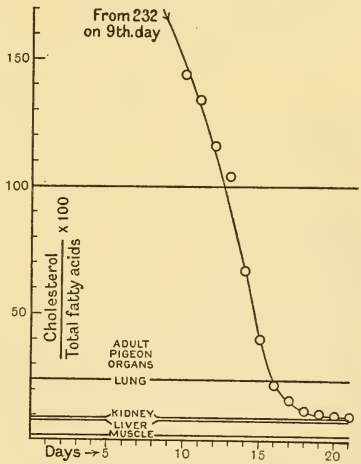



Fig. 392.

is exactly what so many investigators have found. It is instructive to compare this curve for the lipocytic quotient with the standard curve for water-content of the embryo shown in Fig. 220, for not only the general trend, but even the forms of the two curves are the same, both falling rapidly until the 17th day, and then coming to an almost steady state. The conclusion that the two processes are related can hardly be avoided, and it must be admitted that, as far as the embryo of the chick is concerned, Mayer & Schaeffer's generalisation is strongly supported. If the subject were not experimentally rather difficult, it would be interesting to determine the maximum imbibition of water of which the embryonic body is capable each day, and to find out whether it would obey the equation of Mayer & Schaeffer. This has been done in the case of the lung of the foetal sheep by Fauré-Fremiet & Dragoiu (see p. 1573).

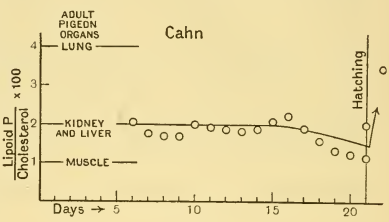


Fig. 393.

The other coefficient which they introduced was:

$$\frac{\text{Lipoid phosphorus}}{\text{Total cholesterol}} \times 100.$$

It ran as follows in adult tissues:

| | Lung | Kidney | Liver | Muscle | Whole body | Investigator |
|---|---|---|---|---|---|---|
| Dog ... ... | 4 | 2 | 1 | 1 | – | Mayer & Schaeffer |
| Rabbit ... | 4 | 3 | 2 | 1 | – | ,, ,, |
| Guinea-pig ... | 3 | 2 | 1 | 1 | – | ,, ,, |
| Pigeon ... | 4 | 2 | 2 | 1 | – | ,, ,, |
| Eel ... ... | – | 2 | 5 | 3 | – | ,, ,, |
| Rat ... ... | – | – | – | – | 2 | Cahn |

Cahn calculated this coefficient from his figures for cholesterol and lipoid phosphorus in the embryonic body, and found it to be very constant throughout development, tending perhaps to fall a little as development proceeded. This is shown in Fig. 393.

## 12·6. Cycloses and Alcohols in Avian Development

As regards ethyl alcohol, which has long been known to be a constant, if minute, component of animal tissues, only one investiga-

tion has been made, that of Aoki, who estimated it on the egg as a whole, using Yamakami's modification of Nicloux's method. His results, which are shown in Fig. 394, led to the conclusion that the alcohol-content of the egg rises steadily until the end of incubation, at which time it has reached an adult value, for the adult fowl has 0·0028 vol. per cent. in its blood and 0·0039 vol. per cent. in its liver. Not the faintest indication is available as to the significance of these results, but Taylor proved in 1913 that alcohol can be found in vertebrate tissues under the most aseptic conditions, and is probably due to the reduction of small quantities of acetaldehyde by the cells. It would be interesting to know more about the metabolism of these substances. Kobert reported in 1903 that crushed eggs of *Testudo graeca*, *Sipunculus nudus*, and *Arbacia equituberculata* would form alcohol from added glucose at 37°. The alcohol was identified qualitatively with the iodoform test, but the sterility of these experiments is doubtful.

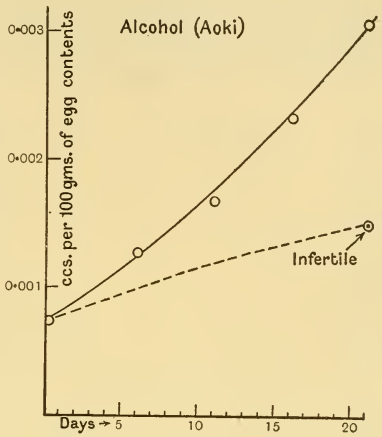


Fig. 394.

The other alcohol with which we are concerned is a very different one, namely, the polyhydric cyclose, inositol, or hexahydroxycyclohexane. In 1908 Rosenberger reported that he had found traces of it in the fresh hen's egg. It was known that on autolysis the inositol content of tissues rose, and the precursor was called inositogen—it is probably a phosphoric ester of inositol, such as the phytin of the plant—and Rosenberger reported that traces of inositogen also were to be found in the fresh egg. Later in the same year, he reversed his opinion on the former point. Then in 1909 Klein was unable to isolate any free inositol from fresh hen's eggs, but got plenty ("eine reichliche Menge") from the chicks at hatching. A couple of years later Starkenstein isolated 20 mgm. per cent. from the yolk of a fresh egg. The question remained in this state until 1924 when

Needham applied a new and approximately quantitative estimation method to the egg during its development. The resulting figures (Black Leghorn eggs) are shown in Fig. 395. It will be seen that the total inositol in the egg rises from 7 mgm. per cent. at the beginning to over 60 at the end. The general shape of the curve is doubly peaked, the first occurring about the 10th day and the second one being coincident with hatching. Between the two maxima there is a depression in which the total inositol descends to a level not greatly

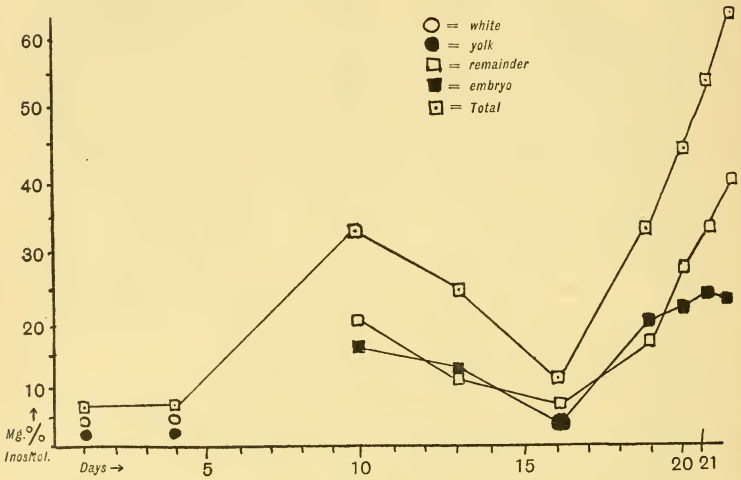



Fig. 395.

above that which it occupied at the beginning. If all the inositol present at the end of development had been in the form of phytin or some similar compound at the beginning, there would be some 62 mgm. per cent. of water-soluble organic phosphorus present at the beginning, while, in point of fact, Plimmer & Scott only found 27 mgm. per cent. of water-soluble organic phosphorus present in the fertilised but unincubated egg. So if phytin were the precursor of inositol in the egg, about three times as much water-soluble organic phosphorus as is actually there would be needed to account for it. Of course, the precursor might be a di- or a tri-phosphate of

inositol, instead of a hexaphosphate, but at present there is no reason for supposing that such compounds exist in the body.

In order to get some insight into the precursor of inositol, Needham injected a series of unincubated eggs with various substances, phytic acid, hexamethylenetetramine (to test the hypothesis of Posternak who suggested that inositol might arise from the condensation of six molecules of formaldehyde) and glucose. Tests showed subsequently

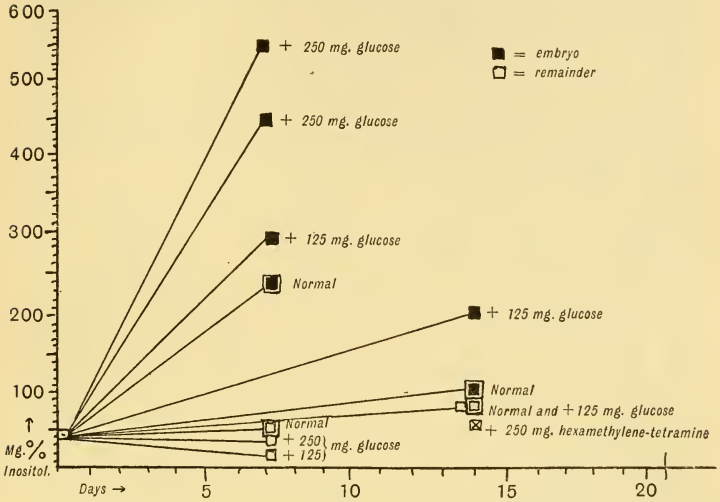



Fig. 396.

the presence of formaldehyde in the egg-interior after injection of hexamethylenetetramine, but neither the eggs which had received phytic acid nor those which had received hexamethylenetetramine could be got to develop normally. Doubtless the highest concentration compatible with normal development could have been found by further searching, but, in view of the positive results obtained with glucose, this was not undertaken. For, as Fig. 396 shows, injection of glucose markedly increased the inositol content of the embryo, though not of the rest of the egg. It would therefore seem likely that the synthesis of inositol from glucose only takes place within the embryo. The existence of this synthesis in the developing embryo agreed with

the conclusions of Greenwald & Weiss, who studied the composition of the urine in dogs after injection of inositol into the circulation, and with those of Needham, Smith & Winter, who studied the behaviour of the free inositol of the body in insulin convulsions. No information is as yet available concerning the way in which the inositol ring is formed from glucose, although, as is well known, rings can be formed by the body, e.g. kynurenic acid. The hen's egg would be a good material to choose in a study directed towards ascertaining the method of synthesis of the cyclose molecule.

## 12·7. Sulphur Metabolism of the Avian Egg

The presence of sulphur in the hen's egg is familiar on account of the deposit of ferrous sulphide at the boundary of yolk and white after long boiling (Tinkler & Soar).[1] The first work which might be included under this heading was that of Liebermann, who estimated the sulphur in the feathers during the last week of incubation, but his method was very questionable, and, as his figures show no definite gradation or constancy, little attention need be given to them. Hopkins' isolation of glutathione in 1921 made it essential to re-open the question of sulphur metabolism in the egg, and he himself observed that in the fresh egg the nitroprusside reaction was quite negative, although the blastoderm and germinal area of a 3 days' embryo gave a brilliantly positive result. This was confirmed by Tunnicliffe. Some years later Murray estimated the glutathione present in the chick embryo at different stages, using Tunnicliffe's method. It was found to be nearly all in the reduced form, and the amounts obtained increased, as was to be expected, with the increase

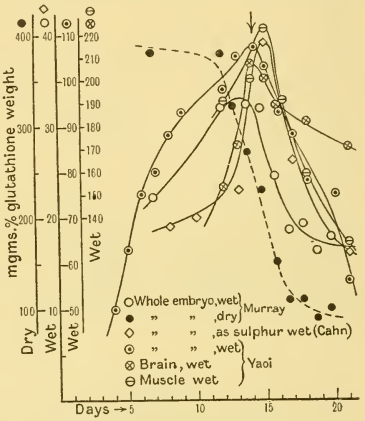



Fig. 397.

[1] And the tarnishing of silver by egg-yolk was known to Pliny: "nigrescit ovi indurati luteo".

in weight of the whole embryo. When related to wet weight the glutathione rose to a peak on the 13th day of development, and when related to dry weight it fell all the time in a more or less S-shaped curve. This is shown in Fig. 397. Later on, Sagara injected glutaminic acid and taurine into hen's eggs at the beginning of development, and observed a rise in glutathione subsequently as they developed; but this was so slight that little confidence can be placed in his conclusions.

Cahn went further into the matter, estimating total, organic, and mineral sulphur in the embryo and the remainder of the egg. His values for glutathione in the embryo did not exactly confirm those of Murray, for the peak on his wet weight curve came a day or two later than the latter's, but nevertheless the two workers are in substantial agreement. A third investigator, Yaoi, has also found the 14th day peak in glutathione per cent. wet weight and was able to trace it in brain and muscle, as well as in the body as a whole.

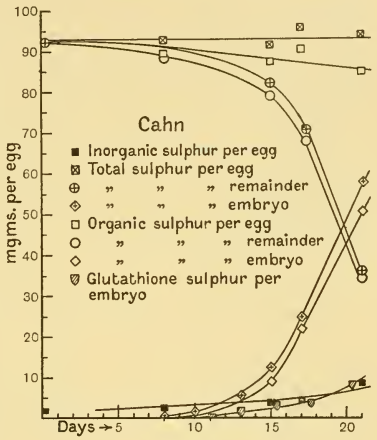


Fig. 398.

Cahn's data for total, organic, and inorganic sulphur are shown in Fig. 398. Apparently the total sulphur in the egg-contents remains at a steady value during development, not receiving any accessions from the shell (though Cahn left this point open). The total sulphur in the embryo rises to about 60 mgm. at hatching, so there is a corresponding fall in the total sulphur of the yolk, the white and the membranes. As the curves for organic sulphur demonstrate, at least 90 per cent. of the sulphur in the embryo and the remainder is in organic form, chiefly, no doubt, combined with the proteins as cystine. In addition to all this, the organic sulphur of the whole egg shows a slow and gradual decline to the extent of some 10 mgm., and there is a corresponding rise in the inorganic sulphur, most of which

seems to be in or associated with the embryonic body. Cahn calculated from his figures the following ratio:

$$\frac{\text{Total organic sulphur}}{\text{Total nitrogen}},$$

which ran as follows:

| Day | Ratio |
|---|---|
| 8 | 8·5 |
| 10 | 7·75 |
| 13 | 8·9 |
| 15 | 8·8 |
| 17 | 8·7 |
| 21 | 8·1 |

Apparently therefore, the total organic sulphur in the embryo grows fairly closely parallel with the total nitrogen. This simple fact may involve so many contributory processes that a trite allocation of significance to it is hardly warranted.

Although strictly speaking out of place here, the work of Thompson & Voegtlin on the rat embryo may be mentioned. As it is the only work on the sulphur metabolism of the mammalian embryo, it can best be taken now. Thompson & Voegtlin estimated the amounts of glutathione by the Tunnicliffe method in rats of different ages, obtaining the following results:

| | Weights in grams | Milligrams glutathione per 100 gm. wet weight |
|---|---|---|
| Embryos ... ... | 0·07– 0·80 | 60 |
| ,, ... ... | 1·04– 1·97 | 58 |
| ,, ... ... | 2·32– 2·95 | 54 |
| ,, ... ... | 3·89– 4·67 | 44 |
| Newly-born ... ... | 4·65– 4·95 | 36 |
| Nursing ... ... | 23·00– 26·00 | 32 |
| Weaned ... ... | 30·00– 50·00 | 31 |
| Adult ... ... | 137·00–170·00 | 23 |

Thus it has been shown both for the chick and for the rat that the concentration of glutathione in the tissues declines with age both during the embryonic and post-embryonic periods.

Targonski, in his work on the allantoic liquid, measured the amount of total sulphur in it. Its amount and concentration, judging from his few analyses, do not seem to be changing in any very definite way, but he calculated the total sulphur/total nitrogen ratio, and found it to be slowly rising, i.e. 0·067 on the 14th day, 0·095 on the 16th and 0·105 on the 18th. It was thus moving in the opposite direction from the total phosphorus/total nitrogen ratio. He could only conclude that towards the end of incubation the breakdown of

nitrogenous sulphur-containing bodies was more intense than at the beginning. The sulphur/nitrogen ratio is shown on the same graph as the phosphorus/nitrogen ratio in Fig. 385.

Somewhat more extensive studies on the sulphur excretion of the chick embryo were made by Takahashi in 1928, who estimated the sulphates in the developing hen's egg, using Folin's method. In the yolk, white and amniotic liquid these were never found, but they were always present in the embryo itself and in the allantoic liquid. Fig. 399, which summarises the results obtained by Takahashi, is of much interest. Takahashi was mainly interested in the ethereal sulphates as an index of the capabilities of the embryo for detoxicating substances harmful to it. As Fig. 399 shows, the total sulphates in milligrams per cent. increase considerably in the allantoic liquid from the 9th day onwards, and this increase is shared equally by the inorganic and ethereal sulphates. It is puzzling that Takahashi found a good deal of total sulphate on the 9th day in the allantoic liquid, but almost no inorganic sulphate, so that, as the difference was taken to be the ethereal sulphate, there was much more of the latter on the 9th than on the 12th day. Takahashi himself seems to have thought that something was wrong with these figures. However, the main outlines of what is happening stand out clearly, for in the embryo itself the total sulphate remains constant, as do its two components, except for the preliminary irregularities which are associated with that just mentioned. Takahashi's conclusion was that ethereal sulphate as a mode of excretion is already in action by half-way through development. But where does the phenol so excreted come from? At that stage there are no bacteria in the gut of the embryo nor would there be much for them to act upon even if they were there.

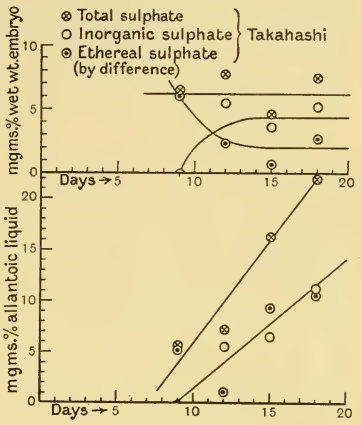



Fig. 399.

The problem had already been raised in the case of man. Senator in 1880 had assured himself that indican and other ethereal sulphates were present in the amniotic liquid but not in the meconium. Then Shibayama ascertained in 1927 that foetal blood gives a more intense indican test than maternal blood[1], although bacteriological examination always shows the meconium to be sterile. *B. coli* does not appear in the intestinal tract until a day or two after birth. The indoxylsulphuric acid must therefore arise in the intermediary metabolism of the foetus, and that its main source always is intermediary metabolism is also the opinion of Kishi who has recently reviewed the question anew.

## 12·8. Phosphorus, Sulphur, Choline and Cholesterol in Reptile Eggs

Kusui, in his work on the cholesterol metabolism of the developing marine turtle, *Thalassochelys corticata*, found, as Fig. 400 shows, a diminution of the total cholesterol and a transfer of it from the free to the combined form. This is extremely interesting in view of what all workers have found to take place in the hen's egg (see Fig. 389) and it would seem as if the increase of esters of cholesterol, at the expense of the free substance, was a phenomenon common to many developing organisms.

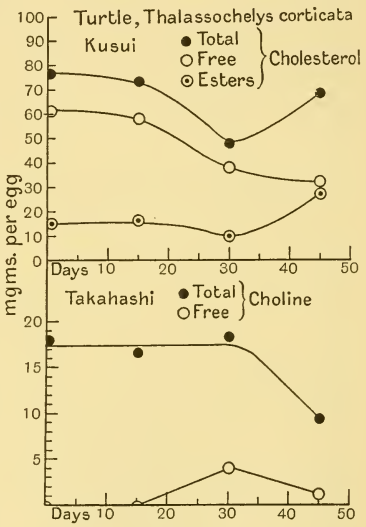


Fig. 400.

Glutathione has also been estimated in these eggs: Tomita found 0·15 mgm. present on the 30th day of development and 0·81 mgm. on the 45th.

The phosphorus distribution is interesting, for just as Plimmer & Scott found a production of inorganic at the expense of organic phosphorus in the hen's egg, so Karashima found the same thing in that of the turtle:

[1] This was confirmed and placed on a quantitative basis by Hensel.

| Days development | Milligrams % $P_2O_5$ in egg-contents | | |
|---|---|---|---|
| | Inorganic | Organic | Total |
| 0 | 6 | 342 | 348 |
| 15 | 13 | 280 | 293 |
| 30 | 15 | 266 | 281 |
| 45 | 123 | 106 | 229 |
| Hatched | 365 | 52 | 417 |

The figures for total phosphorus are insufficiently regular to permit any conclusion as to whether any of it is derived from the shell. The inorganic phosphorus is practically confined to the body of the embryonic reptile, and the organic phosphorus to the yolk. Only minimal amounts of either are found in the egg-white or in the amniotic or allantoic liquids.

The choline has been estimated by Takahashi (see Fig. 400).

## 12·9. Lipoids and Sterols in Amphibian Eggs

If we first enquire as to what is known of the phosphorus distribution in amphibian eggs, we find that Plimmer himself made a step in this direction, collaborating with Kaya in 1909. He used the eggs of the frog (*Rana temporaria*) in two lots: (*a*) ovarian and (*b*) shortly after being laid. It is to be supposed that a certain amount of development had taken place. The figures were as follows:

| | % of the total phosphorus | |
|---|---|---|
| | (*a*) | (*b*) |
| Ether-soluble phosphorus ... ... | 26·2 | 20·2 |
| Total water-soluble phosphorus ... | 4·3 | 11·3 |
| Inorganic phosphorus ... ... | 0·0 | Trace |
| Total protein phosphorus ... ... | 69·5 | 68·4 |
| Phosphoprotein phosphorus ... | 61·9 | 41·0 |
| Nucleoprotein phosphorus ... | 7·6 | 27·4 |

Thus there was every evidence of much the same changes as we have seen take place in the egg of the chick. The ether-soluble phosphorus and the phosphoprotein phosphorus were declining, the nucleoprotein and the water-soluble organic phosphorus were rising. Only the inorganic phosphorus seemed to be maintaining a very low level. This general arrangement fits in with the few later analyses of Parnas & Krasinska, who estimated the lipoid phosphorus only, finding 0·00532 mgm. present in one egg and 0·0039 mgm. in one hatched larva: a loss of 26 per cent. This is not so great a fall as in the chick,

but it must be remembered that hatching occurs very early in the frog. Fauré-Fremiet & Dragoiu found that 25·13 per cent. of the total fatty acids were in the form of phosphatides at the beginning and 20·0 per cent. at hatching.

| | loss by 1 egg (mgm.) | % of initial value |
|---|---|---|
| Unsaturated acids ... | 0·0838 | 30·0 |
| Myristic acid ... ... | 0·0019 | 2·7 |
| Phosphatides ... ... | 0·0444 | 42·0 |

No determinations of lipoid phosphorus were made by Fauré-Fremiet & Dragoiu at the end of the second period, i.e. at the time of disappearance of the yolk-sac. There is general agreement, therefore, that the lipoid phosphorus diminishes during the development of the amphibian egg, but little is known about the behaviour of the other phosphorus fractions.

The cholesterol of the frog's egg has been occasionally investigated. Fauré-Fremiet & Dragoiu found the total unsaponifiable fraction of their ether-alcohol extract to amount to 3·13 per cent. of the total solid present, and this, after treatment with digitonin, separated into 1·43 per cent. of cholesterol and 1·7 per cent. of a body which Fauré-Fremiet identified with the "unsaponifiable X" of Kumagawa, probably spinacene or squalene. Thus the dry weight composition of the ether-alcohol extract at zero hour of development was:

| | % dry weight egg |
|---|---|
| Phosphatides ... ... ... ... | 5·98 |
| Neutral fatty acids ... ... ... | 14·82 |
| Cholesterol ... ... ... ... | 1·43 |
| "Unsaponifiable X" ... ... ... | 1·70 |
| | 23·93 |

Unfortunately Fauré-Fremiet & Dragoiu seem to have forgotten to make any estimations of cholesterol and other unsaponifiable substances at later stages of development. Parnas & Krasinska made determinations of cholesterol only as follows:

| | Cholesterol | |
|---|---|---|
| | % of the total fatty acids | Milligram per individual |
| Egg ... ... ... | 13·6 | 0·056 |
| Hatched larva ... ... | 16·7 | 0·056 |

Thus there was apparently no change in the total cholesterol, although in per cent. of the fatty acids it rose, owing to their utilisa-

tion. This again would agree with what goes on in the hen's egg. The only other researches which have been carried out on the lipoid and sterol metabolism of the amphibian egg are those which have involved histochemical methods alone (e.g. Yamaguchi, who found cholesterol esters in the liver of the toad at hatching). Doubtless the most important of these are the memoirs of Konopacki & Konopacka, and of Hibbard, but it is difficult to tell whether what they call "lipoid" is the same thing as the material known to chemists. It is interesting that Rosenheim finds cholesterol prepared from frog's eggs to be richer in ergosterol than similar products from any other source.

According to Kamiya, no glutathione is synthesised during the development of the frog's egg.

## 12·10. Lipoids, Sterols and Cycloses in Fish Eggs

As regards the fishes, we are almost completely ignorant of what happens to the phosphorus distribution during embryonic growth, but some unpublished work of Rosenheim, Girsavicius, Ashford & Stickland indicates that in the fish egg events occur very like those in that of the hen and the frog. In 1928 they obtained on the trout, *Salmo fario*, the following figures:

| | % of the total phosphorus | |
|---|---|---|
| | Undeveloped eggs | Fry almost at the end of the yolk-sac period |
| Ether-soluble phosphorus ... ... | 26·78 | 0·0 |
| Total water-soluble phosphorus ... | 73·2 | 92·9 |
| Inorganic phosphorus ... ... | 0·0 | 27·9 |
| Organic water-soluble phosphorus | 73·2 | 65·0 |
| Nucleoprotein phosphorus... ... | 0·0 | 7·1 |

If these are compared with those previously given for the frog by Plimmer & Kaya and for the hen by Plimmer & Scott and others, the correspondence will be seen to be considerable. It is evident, however, that in these experiments the ichthulin or phosphoprotein of the trout's egg came out into the watery extract. When this is taken into consideration, it will be seen that the fall in lipoid phosphorus, the rise in inorganic phosphorus, and the probable fall in phosphoprotein phosphorus, parallel events in other eggs.

For cholesterol there is a solitary observation of McClendon's that the unsaponifiable substance soluble in petrol ether formed 1·2 per cent. of the dry weight of the eggs of the brook-trout, *Savelinus fontinalis*, and 1·6 per cent. of the dry weight of its hatched larvae—an increase of 33 per cent. This fraction crystallised, he says, into a

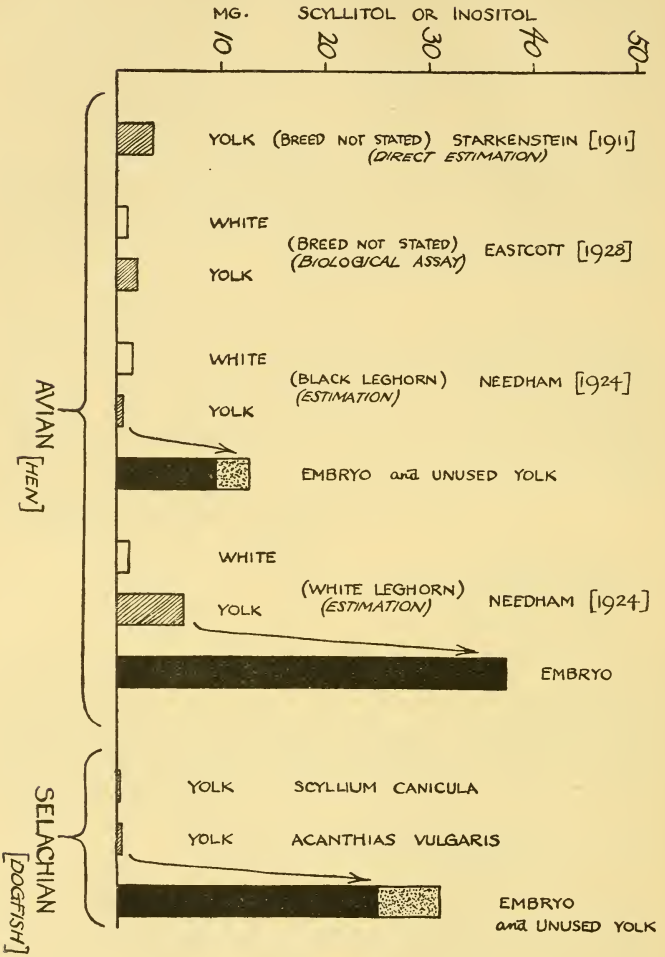
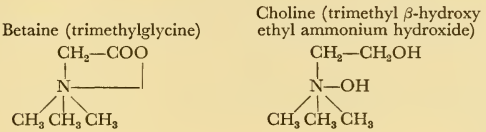

MG. SCYLLITOL OR INOSITOL
10
20
30
40
50
YOLK (BREED NOT STATED) STARKENSTEIN [1911]
(DIRECT ESTIMATION)
WHITE
YOLK (BREED NOT STATED) EASTCOTT [1928]
(BIOLOGICAL ASSAY)
WHITE
YOLK (BLACK LEGHORN) NEEDHAM [1924]
(ESTIMATION)
EMBRYO and UNUSED YOLK
WHITE
YOLK (WHITE LEGHORN) NEEDHAM [1924]
(ESTIMATION)
EMBRYO
AVIAN [HEN]
YOLK SCYLLIUM CANICULA
YOLK ACANTHIAS VULGARIS
EMBRYO
and UNUSED YOLK
SELACHIAN [DOGFISH]

Fig. 401.

solid mass largely composed of cholesterol, but he did not quantitatively separate the cholesterol from the squalene or other unsaponifiable matter.

Nitrogenous bases associated with lipoids have been reported as existing in several kinds of fish embryos. Thus Ackermann & Kutscher isolated in 1907 as much as 2·0 per cent. (dry weight) of betaine from embryos of *Acanthias vulgaris*, and 0·07 per cent. from adult fishes. The formula of this substance shows it to be related to choline:




and it has been believed, though certainly wrongly, that betaine can replace choline in the lecithin molecule. Suwa in 1909 and Kutscher in 1910 have also obtained betaine from embryonic fish muscle. Later Berlin & Kutscher isolated no less than 12 per cent. of betaine (dry weight) from the embryos of *Acanthias vulgaris*, and 0·5 per cent. of choline, as against 0·7 per cent. of choline in the adult muscles and 0·3 per cent. in the adult liver. Nobody has any idea as to the significance of these findings.

Considerable interest attaches to the presence of scyllitol in elasmobranch fishes, for this stereoisomer of the *i*-inositol of animals might or might not be present in the undeveloped eggs. In 1929 Needham examined a number of eggs of *Acanthias vulgaris* and *Scyllium canicula* for scyllitol, using a method as quantitative as possible, and found that only traces were present before development, although at hatching a large amount was to be found. The dogfishes, then, have to synthesise their own particular form of cyclose, just as the birds have to synthesise theirs. Fig. 401 gives a survey of the relationships involved, and it will be seen from it that the cases are indeed quite parallel.

## 12·11. Phosphorus, Lipoids and Sterols in Arthropod Eggs

For insect development we know next to nothing. Tichomirov's balance-sheet of the silkworm egg, made in 1882, old as it is, contains the only information available. He found that the eggs of *Bombyx mori*

contained the following percentages of lipoid (estimation-method not given) and cholesterol:

| | After the diapause | | At hatching | |
|---|---|---|---|---|
| | % wet weight | % dry weight | % wet weight | % dry weight |
| Lecithin ... ... | 1·04 | 2·93 | 1·74 | 5·77 |
| Cholesterol ... ... | 0·40 | 1·12 | 0·35 | 1·16 |

What process explains the rise in lecithin we may well enquire, though at present there is no hope of an answer till more work is done. The cholesterol remains constant, much as in other eggs. Kaneko could not find any cholesterol esters in the silkworm egg by examination in the polarising microscope, but Kitamura and Kamiya affirm that the developing larva, as is the case with the chick, synthesises glutathione:

| | % wet wt. |
|---|---|
| Freshly laid eggs | 0·062 |
| Hatching larvae | 0·088 |

The same remarks apply to the phyllopod crustacean *Artemia salina*, for Hopkins obtained negative nitroprusside tests on the egg-contents and positive ones on the hatched nauplii.

Needham & Needham investigated the phosphorus-metabolism of this brine-shrimp in 1929, obtaining the following figures:

| | Ether-soluble P | Water-soluble inorganic P | Pyro-phos-phate P | Stable water-soluble organic P | Phospho-protein P | Nucleo-protein P | Total P |
|---|---|---|---|---|---|---|---|
| Mgm. per gm. dry weight: | | | | | | | |
| Undeveloped eggs | 0·302 | 0·937 | 1·64 | 0·33 | None | 1·95 | 5·16 |
| Hatched nauplii* | 0·564 | 0·738 | 1·24 | 1·23 | None | 1·41 | 5·16 |
| % of the total phosphorus: | | | | | | | |
| Undeveloped eggs | 5·9 | 18·1 | 31·9 | 6·4 | 0·0 | 37·9 | 100·0 |
| Hatched nauplii* | 10·9 | 14·3 | 24·1 | 23·9 | 0·0 | 27·4 | 100·0 |

* The egg-shells left behind weigh a considerable amount and were included in the weighings. They contain practically no phosphorus.

It is clear from these figures that no absorption of phosphorus takes place from outside the egg, and that there is, as noted in Section 10, a sufficiency of nuclein in the beginning for the needs of the embryo. In view of Tichomirov's results on another arthropod egg, it is of much interest to find the lipoid phosphorus rising. These same

authors also worked with another crustacean, the sand-crab, *Emerita analoga*, obtaining the following results:

| | Ether-soluble P | Water-soluble inorganic P | Pyro-phos-phate P | Stable water-soluble organic P | Phospho-protein P | Nucleo-protein P | Total P |
|---|---|---|---|---|---|---|---|
| Mgm. per gm. dry weight: | | | | | | | |
| Undeveloped eggs | 3·27 | 2·24 | 1·55 | 3·36 | Trace | 1·26 | 11·62 |
| Eye-spots visible | 2·94 | 1·59 | 2·70 | 3·16 | None | 1·25 | 11·65 |
| Embryo larger than yolk | 2·14 | 2·33 | 1·26 | 3·88 | None | 1·50 | 11·10 |
| About to hatch | 1·83 | 2·60 | 2·31 | 3·06 | None | 1·59 | 11·40 |
| % of the total phosphorus: | | | | | | | |
| Undeveloped eggs | 28·1 | 19·3 | 13·3 | 28·9 | — | 10·82 | 100·0 |
| Zoea larvae | 16·0 | 22·8 | 20·3 | 26·8 | — | 13·95 | 100·0 |

Here again, then, we have an egg which is independent of its environment as regards phosphorus and one in which only a small synthesis of nucleins takes place. On the other hand, its lipoid phosphorus diminishes considerably.

## 12·12. Phosphorus, Lipoids and Sterols in Worm and Echinoderm Eggs

Fauré-Fremiet's analyses of the undeveloped eggs of the polychaete *Sabellaria alveolata* showed 1·81 per cent. dry weight of cholesterol and 2·16 per cent. dry weight of "unsaponifiable X", but he made no determinations on hatched worms. On the other hand, in his study of the development of the nematode *Ascaris megalocephala*, he did investigate the distribution of phosphorus before and after the development of the eggs. The phosphorus is here expressed as phosphorus pentoxide.

| | % weight (wet or dry?) | | % of the total phosphorus | |
|---|---|---|---|---|
| | Before | After | Before | After |
| Total phosphorus ... ... ... | 0·90 | 0·90 | 100·0 | 100·0 |
| Inorganic phosphorus ... ... | 0·18 | 0·18 | 20·0 | 20·0 |
| Ether-soluble phosphorus ... ... | 0·23 | 0·28 | 25·6 | 31·1 |
| All other kinds of phosphorus* ... | 0·49 | 0·44 | 54·4 | 49·0 |

* Called improperly by Fauré-Fremiet "nuclein phosphorus".

The inorganic phosphorus remains constant, then, the lipoid phosphorus gains and the rest of the phosphorus loses by some 5 per cent. This state of affairs so contrary to what we find to take place in the hen's egg recalls the similar finding in the case of the silkworm and

the brine-shrimp. But it is difficult to picture what may be going on in the egg of the worm to produce these effects. It is, of course, to be remembered that *Ascaris* is an organism living in very peculiar circumstances[1].

Gephyrean eggs were investigated by Needham & Needham, on the Californian echiuroid worm *Urechis caupo*.

| | Ether-soluble P | Water-soluble inorganic P | Pyro-phos-phate P | Stable water-soluble organic P | Phospho-protein P | Nucleo-protein P | Total P |
|---|---|---|---|---|---|---|---|
| Mgm. per gm. dry weight: | | | | | | | |
| Undeveloped eggs | 2·41 | 1·43 | 1·71 | 1·86 | Trace | 1·39 | 8·8 |
| Trochospheres | 0·32 | 2·40 | 0·27 | 0·13 | Trace | 3·68 | 6·8 |
| % of the total phosphorus: | | | | | | | |
| Undeveloped eggs | 27·4 | 16·3 | 19·4 | 21·2 | — | 15·8 | 100·0 |
| Trochospheres | 4·7 | 35·3 | 3·9 | 2·0 | — | 54·1 | 100·0 |

Here the lipoid phosphorus declines markedly, the total phosphorus receives no accession from outside, and the nuclein phosphorus increases considerably.

When we come to the phosphorus metabolism of the echinoderm embryo, we find ourselves in a region where a good deal of work has been done, yielding at first unsatisfactory and controversial results. Interest in the phosphorus distribution of the echinoderm egg arose in the first instance out of the great increase in nuclear substance during the early stages of echinoderm development, noted by Boveri, Loeb and Godlevski. Thus the ratio of the total volume of the cell to the volume of the nuclear material in the unfertilised egg is 550/1 but in the blastula stage 6/1. There must then, said Loeb, be an increase in absolute and relative amount of nucleoprotein, i.e. in purine bases and nuclein phosphorus, and if the nuclein phosphorus increases, something else must decrease, probably either phosphoprotein or lecithin. Loeb chose the latter substance as being the most likely precursor, and was supported by various investigators, notably Robertson, who wanted the choline from the disintegrating lecithin for his choline surface-tension theory of cell-division. However, the first investigations which were made did not lead to favourable results, for Masing, taking the eggs of *Arbacia pustulosa*, applied the methods of Plimmer & Scott to unfertilised eggs and morulae. Of the unfertilised eggs

[1] The eggs of another parasitic worm (the trematode, *Distomum cygnoides*) are said by Schmidt to contain considerable amounts of esterified cholesterol.

1 gm. of dry substance gave 3·53 mgm. of nucleoprotein phosphorus and no phosphoprotein phosphorus. Fertilised but undivided eggs yielded 3·95 mgm. per gm., and embryos at the morula stage 3·80 mgm. per gm., again with no phosphoprotein phosphorus. 100 mgm. of total nitrogen were associated with 3·6 mgm. of nucleoprotein phosphorus in the unfertilised egg, 4·1 mgm. in the fertilised but undivided egg, and 4·05 mgm. in the morula. From these data, which agreed well among themselves, Masing concluded that there could be no synthesis of nuclein during the period covered by his experiments. Moreover, as has already been mentioned (p. 1157), he isolated the purine bases from his material, and could not find any increase or decrease.

Shackell's work, which was published not long after Masing's, confirmed it. He estimated the ether-soluble and water-soluble organic phosphorus under much the same conditions as Masing, and observed no change in either. He was also unable to find any increase in the nucleoprotein phosphorus, as determined by the protein residue left after treatment in a standard peptic digestion.

Both these papers were severely criticised by Robertson & Wasteneys in 1913, who considered that Masing's material must have been greatly contaminated by the spermatozoa. If this had been the case, his values for the just-fertilised eggs would have been too high, and would perhaps have obscured a real rise in nuclein phosphorus. They also affirmed that the conditions under which his eggs had developed had been inadequate, and that his extraction methods were faulty. Nor did they fail to bring much the same criticisms against Shackell. Later writers, such as LeBreton & Schaeffer, have followed Robertson & Wasteneys in their opinion of the work of Masing and of Shackell, but it must be remembered that Masing published a defence of his methods against the American workers, in which he pointed out that contamination with excess spermatozoa could not explain his results, some of which were derived from unfertilised eggs. Robertson & Wasteneys' own data were very erratic and difficult to interpret as they only estimated alcohol-soluble, water-soluble, and insoluble phosphorus, i.e. mixtures of compounds. They concluded that "the proportion of phosphorus which is present in the form of lecithin, etc., in *Strongylocentrotus* eggs diminishes progressively as development proceeds". Nothing could be said on the basis of their experiments about the nucleoprotein phosphorus.

The subject was evidently ripe for further work. Needham & Needham in 1929, working on the Californian sand-dollar, *Dendraster excentricus*, and on a starfish, *Patiria miniata*, estimated the phosphorus micro-colorimetrically in each of Plimmer & Scott's fractions, either after incorporation of the material with anhydrous calcium sulphate, or after extraction with trichloracetic acid. Table 176 summarises their results:

Table 176.

| | Plaster-of-paris method | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ether-soluble P | Water-soluble inorganic P | Pyro-phosphate P | Stable water-soluble organic P | Total water-soluble P | Phospho-protein P | Nucleo-protein P | Total P |
| *Dendraster excentricus* (mgm. per gm. dry weight): | | | | | | | | |
| Unfertilised eggs | 0·422 | 2·04 | 0·950 | 0·27 | 3·26 | 0·84 | 2·22 | 6·94 |
| Gastrulae | 0·77 | 3·23 | 0·80 | 0·29 | 4·32 | 0·37 | 3·36 | 8·80 |
| Plutei | 0·75 | 3·19 | 1·53 | 1·29 | 6·01 | 0·20 | 2·54 | 9·50 |
| % of the total phosphorus: | | | | | | | | |
| Unfertilised eggs | 6·05 | 29·4 | 13·65 | 3·9 | 46·95 | 12·1 | 32·0 | 100·0 |
| Gastrulae | 8·75 | 36·8 | 9·1 | 3·3 | 49·19 | 4·2 | 38·2 | 100·0 |
| Plutei | 7·90 | 33·6 | 16·1 | 13·6 | 63·30 | 2·1 | 26·7 | 100·0 |
| *Patiria miniata* (mgm. per gm. dry weight): | | | | | | | | |
| Unfertilised eggs | 2·14 | 1·32 | 0·85 | 1·28 | 3·45 | Trace | 1·04 | 6·64 |
| Bipennaria larvae | 1·09 | 1·11 | 1·07 | 1·30 | 3·48 | None | 1·56 | 6·15 |
| % of the total phosphorus: | | | | | | | | |
| Unfertilised eggs | 32·20 | 19·80 | 12·8 | 19·2 | 51·8 | Trace | 15·65 | 100·0 |
| Bipennaria larvae | 17·75 | 18·05 | 17·4 | 21·1 | 56·5 | None | 25·40 | 100·0 |

| | Trichloracetic acid method | | | |
|---|---|---|---|---|
| | Water-soluble inorganic P | Unstable water-soluble organic P* | Stable water-soluble organic P | Total water-soluble P |
| *Dendraster excentricus* (mgm. per gm. dry weight): | | | | |
| Unfertilised eggs | 1·645 | 0·036 | 0·941 | 2·62 |
| Gastrulae | 1·274 | 0·067 | 0·665 | 2·02 |
| Plutei | 0·382 | 0·053 | 1·360 | 1·80 |

* Possibly creatine phosphate.

Attention may be first directed to the rise in total phosphorus which the sand-dollar egg exhibits during its development. The figures given in Table 176, which are the sum of the various fractions, were supplemented by direct estimations of total phosphorus, which demonstrated the same phenomenon, for from an average of 760 mgm. per cent. dry weight before fertilisation, it rose to 990 mgm. per cent.

at the gastrula and 1230 at the pluteus stage. This might be regarded as being due to a removal of other dry substances from the egg by combustion, but such an explanation cannot be correct, for as Ephrussi & Rapkine showed, the total dry solid in echinoderm eggs tends to rise slightly during development owing to the intake of ash from the sea-water. And even if there was a decrease of dry weight it could not, judging from the respiration data given in Section 4·2, amount to more than 5 per cent. of the initial dry weight. Yet the phosphorus increases by 62 per cent. of its initial value.

There is, indeed, nothing against the view that phosphorus is absorbed from the sea-water by certain marine invertebrate eggs, for we know what remarkable accumulations of other elements, such as strontium, can occur in foraminifera. As will be shown, moreover, in Section 13·4, inorganic constituents are absorbed from the sea-water by many marine eggs. If now we ask how the absorption of phosphorus takes place we come upon several points which merit consideration. It is usually surmised that echinoderm eggs hatch so early and the larvae swim about so vigorously owing to the need for wide dispersal of the species in the plankton, but there may also be a chemical reason for this, the egg not being supplied with all that it needs for its proper development and being therefore under the necessity of going to fetch it. An analogous instance among aquatic vertebrates might be found in those teleostean eggs which begin to rotate within their envelopes at a very early stage, setting up intra-ovular currents and more readily absorbing the water which they require. In order to gain some idea of the speed of movement of echinoderm embryos Needham & Needham measured the time taken for them to traverse a course on a micrometer scale, with the following results:

| | | Metres per hour |
|---|---|---|
| *Dendraster* | Blastulae | 1·39 |
| ,, | Plutei | 1·71 |
| *Arbacia* | Gastrulae | 1·17 |
| *Asterias* | Gastrulae | 1·83 |

Assessing thus the average speed[1] at 1·5 metres per hour we may consider an echinoderm gastrula passing for one hour through a tube of sea-water of its own diameter. As this is 0·196 mm. such a tube would contain 4·515 c.c. of sea-water, and in this there would be $1·56 \times 10^{-4}$ mgm. of phosphorus, if we take the phosphorus-content

[1] See also Runnström.

of sea-water to be, for purposes of calculation, 0·034 mgm. per litre. Now during development we find in round numbers an increase of from 7·5 to 12·5, i.e. 5·0 mgm. per gm. dry weight of eggs, and this, reckoning about 4,000,000 eggs to the gram, would be $0{\cdot}012 \times 10^{-4}$ gm. Comparing this estimate with that for the amount of phosphorus in the sea-water, it will be evident that the requirements of the embryo will be amply met by the water with which it comes in contact. In addition to this, turbulence effects must be remembered, and it is probable that under favourable conditions the embryo could dispense with a great deal of its activity as far as the phosphorus-intake is concerned. But if it floated motionless in a perfectly still medium containing phosphorus in as high concentration as sea-water, it is likely that the supply would be deficient.

These facts have a bearing on the problem of the origin of fresh-water fauna. The classical theory of Sollas attributes the paucity of fresh-water species mainly to the custom, usual among marine invertebrates, of hatching early in a larval form and dispersing in the plankton. These minute ciliated larvae, he pointed out, cannot swim against currents of any magnitude and are ill-adapted for river life, so that even if a marine animal solved the osmotic difficulties associated with existence in fresh-water it would see its eggs and larvae swept out to sea again as fast as it produced them. The well-known phenomena of poecilogony (see p. 316) favour this view. But such a theory has no explanation to give for the cases (e.g. the cephalopods) where the embryo spends a long time in its egg before hatching out substantially mature, and yet the group has never penetrated into fresh-water (see p. 317). Now if eggs such as these depend on the salts of the sea-water as a kind of additional yolk, the inorganic environment may become an important limiting factor, and in the case of phosphorus in particular, the margin between supply and demand in normal sea-water may be much reduced. Thus the plankton may use up nearly all the available phosphorus, for as Atkins has shown, there is a seasonal variation in the English Channel, surface water containing 0·0162 mgm. P per litre as a winter maximum and only 0·0032 as the summer minimum, the difference being associated with the growth of the phytoplankton. Corresponding figures for the Clyde area, given by Marshall & Orr, are 0·032 and 0·0022 mgm. P per litre respectively. In the Pacific Ocean the same cycle goes on, according to Stanford and Moberg. Finally, the analyses of Juday,

Birge, Kemmerer & Robinson show that for Wisconsin lake waters, while the dissolved phosphorus may occasionally rise to a typical marine level such as 0·015 mgm. P per litre, it may often be completely absent, and in most cases amounts to about 0·003 mgm. P per litre. Müller again finds hardly detectable traces in the water of Lake Balaton in Hungary. Thus the phosphorus requirements of developing marine eggs may be an important factor prohibiting their colonisation of lake and river waters.

Phosphorus entering an echinoderm egg from sea-water must do so in the form of orthophosphate. Bertolo, using Pollacci's histochemical reaction (which is said to give a blue coloration with all phosphorus compounds, but which in fact only does so with inorganic phosphate), found with *Strongylocentrotus* and *Sphaerechinus* eggs that a blue zone was produced round the periphery. If this observation has any physiological meaning, it must be that the concentration of inorganic phosphate is greatest at the periphery, as would be expected if it were being absorbed through the surface and then turned into something else. Herbst, in his work on the development of echinoderms in artificial saline environments, at first stated (see p. 1273) that phosphorus in the form of calcium phosphate was necessary for normal differentiation and assumed that it was absorbed by the eggs. In a later paper, however, he reported that perfectly normal development would go on in the complete absence of phosphorus and attributed his previous results to the precipitation, and hence the detoxication, of traces of copper in his distilled water. Loeb about the same time also reported that the eggs required no phosphorus from sea-water. Both these workers, however, relied on tests of inferior delicacy, and it is probable that under the conditions used by them, ample phosphorus was available for the developing eggs.

Returning now to Table 176, it can be seen that two methods were used, the first of which (plaster of paris) included the phosphorus of the spicules in the plutei, while the second (trichloracetic acid) did not. Accordingly they give different results from which it can be seen that the phosphorus of the spicules is quite appreciable in amount, being in fact about equal to the total phosphorus taken in from the environment, and almost all in inorganic form. Prenant regards echinoderm pluteus spicules as composed of calcite, and this is especially relevant since calcite originates from an amorphous form of calcium carbonate which tends to be stabilised in the presence of

phosphate when the $P_2O_5/CO_2$ ratio is 0·1. Bütschli, moreover, reports that adult echinoderm spicules contain phosphate.

The distribution of phosphorus in the *Dendraster* egg is, it may be noted, widely different from that in the hen's egg. Instead of the great stores of lipoid and phosphoprotein phosphorus, there is only a small proportion of these bodies, while a prominent place is taken by the various forms of water-soluble phosphorus and nuclein phosphorus. The phospho-protein phosphorus falls during development, perhaps indicating that it is playing a similar part in both cases. The figures for the starfish egg in Table 176 show no absorption of phosphorus from the sea-water, so that the practice cannot be universal among echinoderms, but both organisms provide almost enough nuclein phosphorus in their eggs for the requirements of the embryo. This fact has already been considered in Section 10. The constancy in lipoid phosphorus in *Dendraster* and the fall in *Patiria* may perhaps be related to the fact that the initial amount is small in the former case and large in the latter. Many instances of this have been given in this section, and we may summarise them as follows:

| | Lipoid phosphorus | | Loss of lipoid phosphorus in % of the initial value |
|---|---|---|---|
| | Mgm. per gm. dry weight | % of the total phosphorus | |
| *Dendraster* (Needham & Needham) | 0·42 | 6·09 | 0·0 |
| *Patiria* ,, ,, | 2·14 | 32·2 | 49·0 |
| *Urechis* ,, ,, | 2·41 | 27·4 | 86·6 |
| *Emerita* ,, ,, | 3·27 | 28·1 | 44·0 |
| *Artemia* ,, ,, | 0·30 | 5·9 | 0·0 |
| *Salmo* (Rosenheim, Girsavicius, etc.) | — | 27·0 | 90·0 |
| *Gallus* (Plimmer & Scott) | 5·76 | 64·8 | 70·4 |

This probably indicates two main appropriations of lipoid (*a*) a fundamental quota which no egg can do without, and which is built up into the cell-membranes and other structures of the finished embryo without change, and (*b*) a further store, which in terrestrial eggs may be very great, which is broken down during development yielding phosphorus in a form available for calcification or other uses.

It is interesting to calculate the contribution of phosphorus made to one echinoderm egg by one spermatozoon. In the case of *Dendraster* we found 1 gm. dry weight of egg to contain 8 mgm. of total and 2 mgm. of nucleoprotein phosphorus, and this value may be accepted as being of the same order in various echinoderms. According to Page one million *Arbacia* eggs weigh when dried 0·124 gm., so

that 1 gm. dry weight would contain 8,100,000 of these eggs, or 4,000,000 of those of the sand-dollar. Egg-size is variable:

| | | Diameter in $\mu$ |
|---|---|---|
| Gray ... ... ... ... | *Echinus esculentus* | 73 |
| ,, ... ... ... ... | *Echinus milearis* | 40 |
| ,, ... ... ... ... | *Echinarachnius parma* | 60 |
| Glaser ... ... ... ... | *Arbacia pustulosa* | 74 |
| ,, ... ... ... ... | *Asterias forbesii* | 103 |
| Needham & Needham | *Dendraster excentricus* | 115 |

but may be averaged at 75$\mu$, and spermatozoon size is fairly constant, say 2$\mu$, leaving the tail out of account. The cubic contents would therefore be, of the egg 113,000 cubic $\mu$ and of the sperm 4·18 cubic $\mu$. One egg contains, therefore, $0{\cdot}99 \times 10^{-6}$ mgm. of total and $0{\cdot}25 \times 10^{-6}$ of nucleoprotein phosphorus. Calculation from the relative volumes of egg and spermatozoon, assuming the same water-content, gives 218,000,000,000 sperms contained in 1 gm. dry weight of sperm. The best values for phosphorus-content of echinoderm spermatozoa are still those of Matthews, who obtained 28·6 mgm. phosphorus per gm. dry weight in the case of *Strongylocentrotus*. The total phosphorus in one spermatozoon would thus be $0{\cdot}90013 \times 10^{-6}$ and the nucleoprotein phosphorus could hardly be more than 85 per cent. of this. So the total phosphorus brought in by the spermatozoon which fertilises the egg is about one ten-thousandth part of the total phosphorus which is there already, a computation which agrees roughly with the fact that one spermatozoon is about one thirty-thousandth part of an egg in size.

Little is known from a dynamic point of view about the sterols of the echinoderm egg, although, as has been stated in Section 1, there is a certain amount of information about the sterols as components of the unfertilised ovum. But Ephrussi & Rapkine in 1928 obtained the following figures for the total unsaponifiable fraction of developing *Strongylocentrotus* eggs:

| Hours from fertilisation | % dry weight | % of the total ether extract |
|---|---|---|
| 0 | 3·3 | 15·7 |
| 12 (blastulae) | 3·0 | 16·1 |
| 40 (plutei) | 2·7 | 15·6 |

From this it would seem that the total unsaponifiable fraction always bears a constant relation to the total fatty acids, and as they

decline per 100 gm. dry weight, so does the former. It was made up as follows:

| Hours from fertilisation | "Unsaponifiable X and Y" | | Cholesterol | |
|---|---|---|---|---|
| | % dry weight | % ether extract | % dry weight | % ether extract |
| 0 | 1·5 | 7·0 | 1·8 | 8·7 |
| 12 | 1·3 | 7·4 | 1·65 | 8·7 |
| 40 | 1·3 | 7·5 | 1·4 | 8·1 |

## 12·13. Lipoids and Sterols in Mammalian Development

A certain number of quantitative determinations of lipoid phosphorus in individual tissues have been made, but these will be reserved for consideration in Section 23. Baumann & Holly found 560 mgm. per cent. phosphatides in a 20th-day rabbit embryo and 650 in a 29th-day one, also corresponding values of 240 and 230 mgm. per cent. for cholesterol. Vignes found 205 mgm. per cent. in whole rats (maternal) and 654 mgm. per cent. in their embryos. If we pass to nucleoprotein phosphorus we meet immediately with Masing's work. In 1911 this investigator estimated the nucleoprotein phosphorus in rabbit embryos at different stages of development, but as he used no accurate time scale it is impossible to plot his data. All that can be done is to reproduce the table in which he summarised his results:

Table 177.

| | Nucleoprotein phosphorus associated with 350 mgm. of total nitrogen |
|---|---|
| Embryos ½ to 1½ cm. long, from the 1st half of gestation | 20·3 |
| Embryos 21·5 gm. weight, about 4th week ... ... | 17·4 |
| Embryos a little older than the last lot ... ... | 14·7 |
| Embryos 1 or 2 days before birth ... ... ... | 13·0 |
| Fully developed embryos ... ... ... ... | 12·0 |
| Newly born rabbits ... ... ... ... ... | 11·7 |
| Rabbits 11 days after birth ... ... ... ... | 11·9 |
| Liver from beginning of 4th week ... ... ... | 22·8 |
| Liver from a little later stage ... ... ... ... | 20·4 |
| Livers from embryos 1 to 2 days before birth ... | 18·0 |
| Livers of new-born rabbits ... ... ... ... | 17·0 |
| Livers of rabbits 11 days old ... ... ... ... | 16·0 |
| Livers of rabbits 22 days old ... ... ... ... | 12·0 |
| Adult livers ... ... ... ... ... ... | 10·7 |

The nucleoprotein phosphorus thus decreases relatively to the total nitrogen (i.e. approximately to the dry weight), and correspondingly the nucleoplasmatic ratio decreases. This is in agreement with what has already been said in the Section on purine metabolism.

This is all that we know about the phosphorus metabolism of the

mammalian embryo, if we except, firstly, the researches which have been done on the distribution of phosphorus compounds in the maternal and foetal blood, and which will more conveniently be reviewed in the Section on placental permeability; and, secondly, the suggestion made by Parat that the meconium is not a waste product, but a "véritable embryotrophe". Basing his conclusions entirely on histological evidence, Parat decided that the methods ordinarily used in histology "surprennent la cellule intestinale du foetus humain de 3 à 8 mois en plein travail d'absorption". In other words, the meconium was to be regarded as one of the means of foetal nutrition, though Parat did not explain why the foetus should secrete food for itself. Parat & Delaville subsequently made a few fragmentary analyses of the organic and inorganic phosphorus in the meconium at different stages of development, but the figures were erratic. The subject merits fuller examination than it received from these writers, and might well be joined to a chemical examination of the "uterine milk" (see p. 1467) about which at present we know nothing.

The cholesterol metabolism of the mammalian embryo is again quite uninvestigated, apart from the histochemical observations of Yamaguchi. It is not possible to follow him in his far-reaching conclusions about the functions of cholesterol esters. A certain amount of work has been done on the sterol content of the foetal and maternal bloods, but this will be discussed under the heading of Placental Permeability. The cholesterol content of the foetal suprarenals in man has also been estimated:

| | | % wet weight | | % dry weight | |
|---|---|---|---|---|---|
| | | As esters | Free | Total | Investigators |
| Man | 6th month | — | — | 1·28 | Chauffard, Laroche & Grigaut |
| | 9th month | — | — | 1·34 | ,, ,, |
| | 6th month | 0·053 | 0·227 | — | Beumer |
| | 9th month | 0·043 | 0·247 | — | ,, |
| | 6th month | — | — | 1·335 | Alamanni |
| | 7th month | — | — | 2·172 | ,, |
| | 8th month | — | — | 2·20 | ,, |
| | 9th month | — | — | 4·20 | ,, |

| | Milligrams % dry weight | | | |
|---|---|---|---|---|
| | Suprarenals | Kidney | Liver | Investigators |
| 3rd month | 260 | 214 | — | Chauffard, Laroche & Grigaut |
| 4th month | 329 | 222 | — | ,, ,, |
| 7th month | 816 | — | 250 | ,, ,, |
| 9th month | 1488 | 255 | 251 | ,, ,, |

*END OF VOLUME*

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